Studies on Pigment Production by Microorganisms Using Raw Materials of Agro-industrial Origin

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CERTIFICATE

This is to certify that the thesis entitled ***"Studies on Pigment Production by Microorganisms Using Raw Materials of Agro-industrial Origin"*** submitted by **Tarangini Korumilli** (Roll No-509CH107) to National Institute of Technology, Rourkela in partial fulfillment of the requirements for the completion of the Ph. D. degree in Chemical Engineering, is an authentic work carried out by her under my supervision and guidance.

Dr. (Mrs.) Susmita Mishra

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Abstract

The recent awareness in human safety and environmental conservation has created fresh enthusiasm for natural sources of pigments. Compared to synthetic pigments, microbial pigments shows better biodegradability and higher compatibility with the environment, and have numerous applications from food to cosmetics. Identification of new microbial sources, utilization of low cost substrates and optimization of process parameters are the areas under focus towards economical pigment production. The present study aimed at screening and identification of microbial isolates from soil and water, which are having pigment producing ability. Efforts have been made to cultivate them on numerous cheaper and inexpensive substrates with no special conditions and supplements for effective pigment production. Furthermore, two carotenoid producing strains were also exploited on numerous inexpensive substrates at ambient conditions for pigment metabolites. While analyzing pigment metabolites in all cases, key parameters influencing pigment production by respective strains were optimized by utilizing statistical techniques like Taguchi method and response surface approaches wherever needed. The conditions for enhanced pigment production were established employing microbial isolates and purchase strains individually. The melanin producing *Pseudomonas guinea*, (bacterial strain) was isolated from marine water sample and was employed on vegetable waste for effective pigment production. Another strain of *Bacillus safensis* was isolated from garden soil and showed its ability to produce melanin on fruit waste extract (FWE). It is noteworthy that both melanins produced from marine and soil isolates showed antioxidant, photoprotective and metal ion chelation activities. Addressing garden soil, a new carotenoid producing bacterial strain *Bacillus clausii* was screened and cultivated on FWE for high yield pigment production. The pigment produced by this strain was observed to be a β-carotenoid type and its stability towards thermal treatment was also evaluated. Eying on the significance of carotenoids, microorganisms (*Rhodotorula rubra*, *Xanthophyllomyces dendrorhous*) in their developmental stage were purchased and studied for pigment production on various residues as sole substrates. The obtained yeasts showed improved carotenoids yield i.e. torularhodin and astaxanthin respectively on FWE. In a nut shell we could conclude that there is a huge scope for industrial scale production of Melanin and Carotinoid using easily available agro-industrial raw materials such as rice powder and fruit waste extract (FWE).

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Chapter 1 Introduction

Pigments are the chemical substances that absorb the light of visible region. The produced color is because of the chromophore, a molecule specific structure which captures the sun energy and causes an excitation of electron from external orbital to higher orbital, where the non-absorbed energy is refracted or reflected to be captured by eye [1]. The modern meaning related to the word pigment has its origin in the twentieth century, meaning a substance constituted of small particles which is practically insoluble in the applied medium and is used due to its colorant, protective or other properties. Pigments are compounds with uniqueness of importance to many industries. In the food industry they are used as additives, antioxidants, color intensifiers, etc. Pigments come in a wide selection of colors, some of which are water-soluble. The terms pigment and color are generally applied for the food coloring matters, sometimes indistinctly [2-3]. Until the mid-19th century all colorants were attained from plant or animal extracts. The textile industry used natural pigments, such as cochineal, wood madder, turmeric, or henna. In 1856, H. Perkin established the first factory of organic synthetic colors to produce mauve. A few years later the discovery of diazotization and a coupling reaction by Peter Griess was the next major step forward for development of the color industry. In the 19th century, synthetic organic dyes were developed, creating a more

economical and broader range of colorants. Since then their quality has been enhanced due to extensive research and development [4-5]. The economic consequence of the color industry is clearly reflected in the large number of synthesized compounds; as many as 700 colorants are currently available. They have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes; encompass various hazardous effects [6]. All synthetic food components suffered severe criticism, including synthetic additives and predominantly food pigments. Today, all food color additives are cautiously regulated by federal authorities to ensure that foods are safe to eat and accurately labeled [3-5]. Pigments produced from natural sources are of worldwide interest and is gaining significance. These are looked upon for their safe use as a natural food dye in substitute of synthetic ones in spite of having of undesirable market [4]. It is therefore, essential to explore various natural sources of food grade pigments and their potentials [5]. The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes has been mounting in recent years [7-8-9]. Natural colorants or dyes derived from flora and fauna are believed to be secure because of non-toxic, non-carcinogenic and biodegradable in nature. Natural pigments are attained from ores, insects, plants and microbes [10-11].

Among all, microbial pigments are dominant sources. The microbial production of carotenoids, pigments from vegetables or chemical synthesis, have problems of seasonal and geographic variability in the production and marketing [21].The microbial production of carotenoids, They are of great interest owing to the stability of the pigments produced and the accessibility of cultivation technology [2-3, 7-8, 12-13]. The advantages of pigment production from microorganisms comprise easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. The economic

advantages of microbial pigments include growth on natural substrates such as red rice wine, red bean curd as carbohydrate source [21-22]. Microbial colorants are in use in the fish industry already, for example to improve the pink color of farmed salmon [12-15] In nature, color rich and pigment producing microorganisms (fungi, yeasts, and bacteria) are fairly common. Microorganisms produce various pigments like carotenoids, melanins, quinones, flavins, prodigiosins and more specifically monascins, violacein or indigo [7-8, 16-17].

Carotenoids such as β-carotene and xanthophylls like astaxanthin play central roles in the metabolism of the eye's macula and retina and in retaining healthy vision. β-carotene play a constructive role in the prevention of cancer and as chemo-protectives [7] [18-19]. In addition, it, also act as neutraceutical that avert carcinogenesis through anti-oxidative, anti-free radical or other mechanisms [20].

The production of microbial pigments is very much affected by the temperature of incubation, depending upon the type of microorganism [23-24]. The growth of *Monascus* sp. entails 25-28 °C for the production of pigment, whereas *Pseudomonas* requires 35-36 °C for its growth and pigment production [25]. pH of the medium is another parameter that affect the growth and kind of pigment produced by the in which microorganisms are grown [25]. The yield of astaxanthin from *Phaffia rhodozyma* was 325 to 212 µg/g astaxanhin at a pH of 6.5 to 3.5 [26]. Pigment production is also affected by carbon source like glucose, fructose, lactose, maltose, galactose, etc [27-26] and nitrogen source depending upon the microorganism [28]. Minerals also has significant role in pigment production [29]. Zn ($2x10^{-3}$ M and $3x10^{-3}$ M) inhibited the growth in liquid medium whereas in solid medium vigorous growth and pigmentation was observed.

The optimization of growth conditions of microorganisms, particularly physical and nutritional parameters are of prime importance in the development of any pigment production process owing to their impact on the economy and practicability of the process. Medium optimization and physical conditions have been customarily performed using one-factor-at-a-time method. The disadvantages of such a classical method are that it is time consuming, laborious and expensive; in addition, it fails to resolve the combined effect of different factors [30-31]. Eyeing on maximizing the pigment yield, productivity and minimizing the production costs, most of the recent optimization efforts have relied on statistical experimental design and response surface analysis [32] and, to a smaller extent, artificial intelligence techniques such as genetic algorithms [33]. Statistical design is a potent tool that can be used to account for the main as well as interactive influences of fermentation parameters on process performance. It is an efficient way to generate useful information with limited experimentation, thereby limiting the process development time and cost [34]. Therefore, researchers are encouraged to apply statistical experimental approaches such as Taguchi method [35] and response surface methodology (RSM), which provide a great amount of information based on only a small number of experiments [31].

On summing up, the growing apprehension over the eventual harmful effects of synthetic colorants on both the consumer and the environment has raised preferential interest in natural coloring alternatives [5]. Among all, microbial colorants popularly known as pigments have some advantages over plant and animal based colorants Extensive studies proved that microbes are known to produce a large amount of stable pigments. [8, 36]. Large amounts of agro-industrial and domestic residues are generated from diverse economic activities; utilization of these residues as inexpensive substrates to support the growth of

microorganisms to generate value-added products like pigments are of biotechnological interest in recent years [8, 37]. Several processes and methodologies have been developed and developing that utilizes a variety of cheaper substrates and wastes as alternative substrates for the production of microbial pigments [38]. The utilization of several wastes as or raw materials notably helps in solving pollution problems, while their disposal may otherwise cause. In addition to the above, screening of different resources, establishing simple methodologies, and exploring the microbial synthesis of pigments on inexpensive substrates is an attractive option to develop commercial scale production [2, 37-38]. Aiming at natural pigments on readily available agro-industrial materials, this study mainly focus on 1) Isolation of pigment producing microorganisms and the production of pigments (especially carotenoids and melanins), 2) Simple production of commercial pigments in higher amounts by purchased strains (which are at developmental stage) and 3) Optimization of key parameters influencing pigment production where ever necessary

1.1 Motivation and scope

The use of synthetic organic colors has been acknowledged for many years as the most reliable and economical method of restoring some of the food's original shade to the processed product. Synthetic colors are superior to natural pigments in tinctorial power, ease of application, stability, and cost effectiveness. However, from the health safety viewpoint they are not accepted by consumers, so over the past years growing interest in natural food colorants has been observed [5].

The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing practices has been increasing in recent years [24]. Natural

pigments can be obtained from three major sources i.e. animals, plants [25] and microorganisms [13]. The accessible authorized natural pigments from animals and plants have numerous drawbacks such as limited range, volatility against light, heat or adverse pH, low water solubility and are often non-availability throughout the year. Moreover microbial pigments are of great interest owing to the stability of the pigments produced and the availability of cultivation technology [17], [26]. The benefits of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, microbial pigment production is now one of the promising and emerging fields of research to reveal its potential for various industrial applications [13], [27], [28].

From an industrial point of view, there is a necessity to develop a high throughput and cost-effective approaches for large scale production of various microbial pigments. Conventional media used for the biosynthesis of microbial pigments are rich in a variety of nutrients. Microorganisms vary in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources e.g. (glucose, sucrose, and fructose) is expensive from cost-effective casing, so the industrial processes try to use contemptible carbon sources especially industrial wastes, variety of plant seed oils etc. have also been used as carbon substances for obtaining different pigments. From an industrial point of view it is essential to obtain a suitable medium to simultaneously improve the growth of organism and the pigment production [17], [29], [30].

Thus, there is an urgent need for alternative colorants that are natural, cost effective and easily degradable and without production of recalcitrant intermediates when they enter the environment. There is an increasing interest involving microorganisms as a possible

alternate source of colorants used in food, cosmetic and pharma industries. In this direction, the exploration of several wastes as substrates for the production of microbial pigments could make huge cut-off in the production costs of these natural biocolorants and makes the approach promising and worthwhile.

1.2 Organization of thesis

The prime objective of the work presented in this thesis was to select potent pigment producing microorganisms from natural sources like marine water and soil. Selections of suitable cheaper substrates for economical productivity of commercial pigments, development of process by optimization of key parameters are subsequent accomplishments. Along with above utilization of cheaper substrates for improved pigment production by carotenoid producing yeast strains is also investigated.

The thesis has been organized into five chapters. **Chapters 1** and **2** represent introduction to the topic and relevant literature review regarding microbial colorants. History of colorants, significance of dyes and pigments, various classes of pigments, scope of microbial pigments, available technologies and practices etc., are provided in these chapters. The extensive summary and descriptions of **Chapters 1** and **2** provided ample motivation and facilitated in framing the research objectives of the work.

Chapter 3 addresses the key role of melanins and carotenoids which are at research project and development stage pigments and connects the coming chapters as a bridge by justifying the objectives of the work. This chapter deals with the key issues like sampling, screening, isolation of pigment producing microbes, synthesis of commercial pigments by using purchased strains, microbial cultivation strategies, selection of suitable substrates etc.

Chapter 4 deal with approach and investigations of the work and is mainly divided into two parts **part I** and **part II**. Studies pertaining to the isolation of pigment producing bacteria and the production of pigments on cheaper substrates were encompassed in **Part I** with sub-chapters of **4.1, 4.2** and **4.3**. Sub-chapters **4.1** and **4.2** deals with the isolation of melanin producing strains from natural resources (like marine water and a soil sample), pigment production on cheaper substrates like vegetable waste and fruit waste extract, optimization of key production parameters, analysis of the obtained melanins, evaluating their efficiency as photoprotective, radical scavenging and metal chelating agents. Additionally, carotenoid producing activity on rice powder as a sole substrate was described in sub-chapter **4.3** by a novel garden soil isolate. The pigment was subjected to various analysis and the influential production parameters were optimized using a simple Taguchi approach.

Part II of Chapter 4 detail facile synthesis of commercial pigments by purchased strains on cheaper substrates with sub-chapters **4.4** and **4.5**. Sub-chapter of **4.4** illustrates the carotenoid production by the obtained yeast strain *Rhodotorula rubra* and its ability to utilize the FWE as sole substrate for pigment production. This study employs a simple two step optimization of key parameters involved in production. Furthermore, the sub-chapter **4.5** presents production of the carotenoid astaxanthin by yeast *Xanthophyllomyces dendrorhous* and the process of optimization of key parameters using FWE. Antioxidant assay of the obtained carotenoids by both the yeasts were also evaluated in respective studies.

Finally, **Chapter 5** summarises, major findings of all the chapters and suggestions for further work in the arena of commercial scale pigments production on cheaper substrates using microorganisms in particular.

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Chapter 2 Literature Review

2.1 Outline

Pigments are the colors that we observe at each step of our lives, because pigments are present in all the organisms in the world, where plants are the principal producers. Pigments are present in leaves, fruits, vegetables, and flowers; also, they are also found in skin, eyes, and other animal structures; and in bacteria and fungi. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics, and in other products. However, natural pigments have important functions besides imparting beauty, such as photosynthesis by chlorophylls and carotenoids. Respiration in animals by hemoglobin under stress conditions plan synthesizes flavonoids; the quinones play very important role in the conversion of light into chemical energy. The melanins act as a protective screen in humans and other vertebrates, and in some fungi melanins are essential for vital cycles. Pigments have a well-known pharmacological activity such as anticancer and effective against cardiovascular diseases [1-2].

In the recent years, pigments produced from natural sources are of worldwide interest and is gaining importance. The demand for natural source of pigments is increasing day by

day because of the consciousness of positive health benefits out of natural compounds [1]. It is therefore, necessary to explore various natural sources of colorants and their potentials [2]. Though many natural colors are available from ores, insects, plants and microbes; microbial colorants play a significant role as food coloring agent, because of its production and easy down streaming process [3-4].

2.2 History of colorants

Until the mid-19th century all dyes were obtained from animal or plant extracts. The textile industry used natural pigments, such as turmeric, cochineal, wood madder, or henna. In 1856, H. Perkin established the first industrial unit of organic synthetic dyes to produce mauve. A few years later the discovery of diazotization and a coupling reaction by Peter Griess was the next major advance for development of the color industry [5]. In the 19th century, synthetic organic dyes were developed, creating a more inexpensive and wider range of colorants. Since then their quality has been enhanced due to extensive research and developments. The economic significance of the color industry is clearly reflected in the large number of synthesized compounds; as many as 700 colorants are currently available [4].

Toward the end of the 19th century, when synthetic colors were first adopted for use on a large scale, they were hailed as a considerable technological breakthrough. The term 'synthetic' was associated with the idea of progress and synthetic colorants were actually considered safer in food than the naturals, as they were tinctorially much stronger and consequently a smaller quantity was needed to achieve a specific colored effect [5].

Synthetic colorants were used in foods, medicines, and cosmetics, but through the years their importance reduced. This cutback of synthetic colorants started about five decades

ago. All synthetic food components suffered severe criticism, including synthetic additives and mostly food pigments. Color additives were one of the first man-made (synthetic) products regulated by law. Today, all food color additives are cautiously regulated by federal authorities to ensure that foods are safe to eat and accurately labeled [6].

2.3 Dyes and pigments – their implications

Color is an important marker of food quality. The consumer links food color with good processing and safety. However, color cannot be studied without taking into account the human sensory system. Perception of color is associated to three factors: spectral composition of the light source, physical object characteristics, and eye sensitivity [7]

Coloring agents can be divided primarily into two classes i.e. pigments and dyes. Dyes are water soluble substances and have at least one salt-forming group. The most common is the sulfonic acid group; however carboxylic acid residues can also be used. These dyes are generally isolated as sodium salts. They have colored anions and are well-known as anionic dyes. The other dyes containing basic groups, like $–NH_2$, $-NH-CH_3$, or $–N(CH_3)_2$, from water –soluble salts with acids. These are the cationic dyes and have positively charged colored ion. If both acidic and basic groups are present, an internal salt is usually formed [8]. Pigments are the particulate solids disperse into a medium without significant solution or their interactions. They are oil-soluble or solvent-soluble colorants lack with salt-forming groups. They occupy a major place in our daily life. Pigments are used in food, cosmetics, paints, pharmaceuticals, glass, textiles etc. The most primitive known pigments were natural minerals. Natural iron oxides, anhydrous Fe_2O_3, charcoal and so on are several well-known pigments since prehistoric times [9].

The color of material such as food is the outcome of the presence of natural pigments or of added synthetic organic dyes. The definition of a natural colorant is variable from one country to another, but generally, natural colorants comprise the pigments occurring in unprocessed food, and those that can be formed upon heating, processing, or storage [5]. These may be isoprenoid derivatives like carotenoids, porphyrins like chlorophylls and hemes, phenolics like anthocyanins, betalains, quinones, curcuminoids and some miscellaneous naturally occurring colorants like riboflavins, caramels, melanoidins, melanins and so on [2, 5]. Based upon major sources, natural pigments may be divided into three major classes which include plant, animal and microbial groups. All natural pigments are unstable and participate in different reactions and their color is robustly dependent on conditions of storage and processing.

Organization of pigments can be done in numerous w ays [2] and can be stated as follows:

By Their Origin

Pigments can be classified by their origin as natural, synthetic, or inorganic. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Synthetic pigments are obtained from laboratories.

By the Chemical Structure of the Chromophore

Also, pigments can be classified by taking into account the chromophore chemical structure as Chromophores with conjugated systems: carotenoids,anthocyanins, betalains, caramel, synthetic pigments, and lakes. Metal-coordinated porphyrins: myoglobin, chlorophyll, and their derivatives.

By the Structural Characteristics of the Natural Pigments

- Moreover, natural pigments can be classified by their structural characteristics as:

- Tetrapyrrole derivatives: chlorophylls and heme colors.
- Isoprenoid derivatives: carotenoids and iridoids.
- *N*-heterocyclic compounds different from tetrapyrroles: purines, pterins, flavins, phenazines,
- Phenoxazines, and betalains.
- Benzopyran derivatives (oxygenated heterocyclic compounds): anthocyanins and other flavonoid pigments.
- Quinones: benzoquinone, naphthoquinone, anthraquinone.
- Melanins.

As Food Additives

By considering the pigments as food additives, their classification by the FDA is

Certifiable: These are manmade and subdivided as synthetic pigments and lakes.

Exempt from certification: This group includes pigments derived from natural sources such as vegetables, minerals, or animals, and manmade counterparts of natural derivatives.

2.4 Biological pigments- different classes

2.4.1 Biological pigments

In recent times growing concern on the use of edible coloring agents has banned various synthetic coloring agents which have a potential of carcinogenicity and teratogenicity [10]. Biological pigments are also well-known as pigments or biochromes. These are the constituents produced by living organisms that have a color resulting from selective color absorption. The topic of synthetic dyes in food is in conversation for many years. The study and negative valuation of synthetic food dyes by the modern consumer have raised a strong

interest in natural coloring substitute. Nature is rich in colors (minerals, plants, microalgae, etc.), and pigment-producing microorganisms (fungi, yeast, bacteria) are reasonably common [11-12]. All biological pigments selectively absorb particular wavelengths of light while reflecting others. The light that is absorbed may be used by the organism or plant to power chemical reactions, while the reflected wavelengths of light govern the color of the pigment that will appear to the eye. Biological pigments based on the source were categorized as mentioned below.

2.4.2 Plant pigments

Several companies decided to color the food with plant extracts or pigments from plants. Plant pigments include a variety of diverse kinds of molecules including porphyins, carotinoids, anthocyanins and batalains. In plants these pigments will also assist in pollination by attracting the pollinators [13]. Some major plant pigments are as follows [2-3]:

a) ***Chlorophylls***

These are the principal pigments in plants. These porphyin compounds absorb yellow and blue wavelengths of the light and reflect the green. All land plants, green plants and green algae possess two forms of this pigment chlorophyll *a* and chlorophyll *b*, whereas red algae possess only chlorophyll *a*.

b) ***Carotenoids***

These are the orange, red or yellow tetraterpinoids. They gather the wave lengths that are not generously absorbed by the chlorophylls, most familiar carotenoids are carotene, lutein, and lycopene. Carotenoids have been shown to act as natural antioxidants

c) ***Anthocyanins***

These are water soluble flavonoid pigments that look like red to blue according to the pH. The anthocyanin catches the light that has passes the leaf and reflects it back to the regions having chlorophyll in order to maximize the use of available light. Color range of pink/red to mauve/blue were obtained from elderberries, black grape skin, black carrots, red cabbage etc.

d) Betalains

There are water soluble pigments like anthocyanins but unlike anthocyanins they are indole-derived substances synthesized by tyrosine**.** These classes are only found in caryophyllales (including cactus and amaranth) and never co-occur in plants with anthocyanins. These are responsible for the deep red color of beets and are used as food-coloring agents commercially.

Although plant pigments are emerging alternatives, they suffer from several bottlenecks i.e. for example; abundant orange-yellow pigment like curcumin (from plant rhizome of *Curcuma longa*) has to be debittered to avoid its odour and sharp taste. And pigments like anthocyanins, chlorophyll, betanin are pH-dependent, oxygen sensitive, heat sensitive, and subject to photo-oxidation some limitation of must be [14].

2.4.3 Animal pigments

Our ancestors managed to obtain wide range of pigments from animal sources before chemical equivalents are manufactured the most rare and difficult to obtain became symbol of wealth and status for example the color purple is associated with wealth and royalty. The purple dye of the ancients is one of the old pigments known in 13^{th} century. Murexes types of drilling snails have a mucus secreting organ called hypobranchial gland. Tyrian purple is

eked out in small amount from the mucus of certain mollusks. Similarly caramine made from cochnieal insects is much more concentrated than the traditional red dye obtained from madder root. Carminic acid and carmine of orange to red and pink to red comes from a female cochineal insect from Peru and Equador. The pigment is price-sensitive here. Dye from kermes and cochneal was of high demand throughout Europe as it is used to color the fabrics of royalty, nobility and church leaders. For several centuries it was the dye used in British red coats, hand woven rugs, as paint. Carmine derived from cochneal is used to color drinks, foods, meat, sausages, processed poultry products, bakery products, pie fillings, icings, jams, desserts, yogurt, cheese, ice creams and other dairy products. Cosmetic industry is the major consumer of insoluble carmine pigment, particularly for hair and skin products, lipsticks, face powders, rouge and blushes. Another major application is in pharmaceutical industry to color ointments and pills. Lac insect produces red dye similar to that of the caramine dye, the water soluble red dye comes from the body of the insect is obtained by aqueous extraction and is processed into seedlac and shellac. They are used in a multitude of applications including varnishes, paints, printing inks, sealing wax, coat pills, sweets and chocolates. Shellac is used in making vinyl records and to color Indian military uniforms and found in oriental carpets [14]. Other pigments like Canthaxanthin of orangish pink color are obtained from salmon, shrimp and flamingos. While several animal pigments are price sensitive, many area having the main disadvantages such as limited color range and availability [14].

2.4.4 Microbial pigments

There are a number of natural pigments but only a few are available in adequate quantities for industrial production. Production of pigments from microorganisms is beneficial over other

sources because microorganisms can grow rapidly which may lead to a high productivity of the product [15-16].

a) Fungal pigments

Many fungi produce pigments during their growth which are substantive as specified by the permanent staining that is often associated with mildew growth on textiles and plastics. Some fungal pigments have been shown to be anthroquinone derivatives, resembling the important class of vat dyes. Fungal compounds therefore have potential for the direct production of textile dyes or dye intermediates replacing chemical synthesis. The production and evaluation of microbial pigments as textile colorants is currently being explored [17].

b) Bacterial pigments

Some bacteria produce pigments which can be observed after they grow into colonies. Pigments can aid to identify bacteria. For example certain bacteria produce water soluble pigments which spread through the medium in which they grow. Others give pigments that are soluble in fat [18]. The yellow pigment from zeaxanthin from *Flavobacterium* species can be used as an additive in poultry feed to fortify the yellow color of the skin of birds or to accentuate the color of the yolk of the egg. A yellow pigment zeaxanthin from *Flavobacterium* sp. can also be used in cosmetic and in food industry. Canthaxanthin from the photosynthetic bacterium *Bradyrhizobium* sp. has been used in fish feed for numerous years. Halobacterium is also one more source of canthaxanthin. astaxanthin from *Agrobacterium aurantiacum* [19].

c) Yeast pigments

Some yeast can produce valuable carotenoids in pure culture on low-cost substrates, thereby providing an alternative to chemical synthesis. Asthaxanthin from *Xanthophyllomyces dendrohous* formerly known as *Phaffia rhodozyma* is a carotenoid pigment which is used as a food colorant and widely used in the animal feed to impart color to the animal skin as animals have no capacity to synthesis the carotenoid pigment. Hundreds of scientific papers and patents deal with asthaxanthin production using this yeast and pigment production process has not been economically efficient till now. Most of the research in recent years is also focused on *Rhodotorula glutinis* which gives carotenoid pigment. However some papers reported reasonable pigment production with other species such as *R. gracilis*, *R. rubra* (now *R. mucilaginosa*) and *R. graminis* too. The main compounds obtained by these red yeasts are torulene and torularhodin with minute quantity of beta carotene [16, 19].

Among all the stated pigment sources, microbes have vast potential to produce diverse bioproducts and one such bioproduct is pigments. Interest in microbial pigments has increased considerably, mainly due to the benefits to human health and also to the growth of certain areas such as agriculture, especially aquaculture and poultry industry [20], Britton, nutritional supplements, food industry where they are used as coloring agents for cooked sausages, soft drinks, baked goods and pharmaceutical as additive to cosmetics. For example, carotenoids market has resulted interesting in 2010 estimated at nearly $1.2 billion, but the expectations for 2018 are increasing considerably supposing to reach $1.4 billion with a compound annual growth rate of 2.3 [21].

The production and application of microbial pigments as natural colorants has been studied by various researchers and is one of the emerging fields of research [14-15, 18-19, 21-22]. Most of the microbial pigments productions are still at the R&D phase. And there are many studies in the literature on various microbial pigments which focus on production and application of specified pigment in each case.

Efforts have been made in order to ease the production costs of microbial derived pigments compared to those of synthetic pigments or pigments extracted from natural sources. Innovations will progress the economy of pigment production by isolating new or creating better microorganisms, by improving the processes. Hence, work on the microbial bacterial pigments should be excelled especially in finding cheap and suitable growth mediums which can reduce the cost and increase its applicability for industrial production [15].

2.5 Significance of microbial pigments as natural colorants

Microorganisms are the most prevailing creatures in existence and determine the life and death on this planet. Microorganisms are associated with all the foods that we eat and are accountable for the formation of certain food products by the process of fermentation and can also be used as a source of food in the form of single cell proteins and food supplements in the form of pigments, amino acids, vitamins, organic acids, and enzymes.

In this way the pigments from microbial origin are a good alternative. Microorganisms are known to produce a range of pigments; therefore they are promising source of food colorants [23-24]. Some of the most significant natural pigments are carotenoids, flavonoids, tetrapyrroles and some xanthophylls as astaxanthin. The pigment

most frequently used in industries is beta-carotene which is obtained from some microalgae and cyanobacteria. Astaxanthin produced from *Phaffia rohodozoa* and *Haematococcus pluviais,* is a red pigment of great commercial value and is used in feed, pharmaceutical and aquaculture industries. Microorganisms which have the capacity to produce pigments in high yields include species of *Monascus*, *Paecilomyces, Serratia, Cordyceps*, *Streptomyces* and yellow-red and blue compounds produced by *Penicillium herquei* and *Penicillium atrovenetum*, *Rhodotorula*, *Sarcina*, *Cryptococcus*, *Monascus purpureus, Phaffia rhodozyma*, *Bacillus* sp., *Achromobacter, Yarrowia* and *Phaffia* also produce a large number of pigments [16, 21].

Most of the bacteria and fungi are extensively studied for their potential as a source of food colorants. Natural pigments possess anticancer activity, contain pro-vitamin A and have some important properties like stability to light, heat and pH [25]. Thus, the food industry has become increasingly interested in the use of microbial technology to produce colors for usage in foods. It can also help to overcome the growing public apprehension over the adverse health effects of addition of synthetic colors in food products.

Furthermore, natural colorants will not only be valuable to the health of human beings, but it will be a benefit for the preservation of biodiversity as harmful chemicals released into the environment while producing synthetic colorants could be stopped. These natural colorants are used in baby foods, breakfast cereals, sauces, pastas, processed cheese, fruit drinks, vitamin-enriched milk products, and some energy drinks. Thus, natural colors in addition to being eco-friendly, can also serve the dual need for visually appealing colors and probiotic health benefits in food products [26].

2.6 Classification of microbial pigments

Pigments produced by organisms as reminiscence of its secondary metabolism are commonly mentioned as biopigments. These biopigments have extensive synthetic and commercial application [27]. Biological pigments can be categorized based on structural affinities and natural occurrence. Some examples of naturally occurring microbial pigments are:

Riboflavin: It is a yellow water-soluble vitamin produced by various microorganisms. Traditional chemical synthesis of riboflavin is now being exchanged by commercially competitive biotechnological processes using ascomycetes *Ashbya gossypii*, filamentous fungi *Candida famata*, or bacterium *Bacillus subtilis* [28]. It is used in baby foods, breakfast cereals, sauces, pastas, processed cheese, fruit drinks, vitamin-enriched milk products, and some energy drinks.

Beta-carotene: Phycomyces and *Mucor circinelloides* (wild type) are a prospective source of beta-carotene. The European Union Committee considers that beta-carotene obtained by fermentation of *Blakeslea trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a coloring agent in foodstuffs [19].

Canthaxanthin: It is produced as the major carotenoid pigment by orange and dark pink-pigmented bacteriochlorophyll containing *Bradyrhizobium* (photosynthetic) strains isolated from stem nodules of *Aeschynomene* species and *Halobacterium* sp [29]. Canthaxanthins are effective antioxidants and inhibit the oxidation of lipids in liposomes [30].

Carotenoids: These are yellow to orange-red pigments that are ubiquitous in nature. Several numbers of microorganisms produce this pigment such as *Serratia* and *Streptomyces.* Carotenoids are potent antioxidants and are widely used as food colorants. Majority of

microbes investigated produce carotenoids belonging to *Myxococcus* [31], *Streptomyces* [32], *Mycobacterium*, *Agrobacterium* and *Sulfolobus* [33].

Prodigiosin: It is a multipurpose red pigment, produced by a number of microorganisms such as *Serratia marcescens, Vibrio psychoerythrus, Rugamonas rubra,* actinomycetes, such as *Streptoverticillium rubrireticuli* and other eubacteria [26]. It is known to have anti-malarial, antibacterial, antineoplastic and antibiotic activity.

Phycocyanin: It is a blue pigment, produced by cyanobacteria which contain chlorophyll *a*. The blue colorant is known by the name spirulina (blue green alga), and is used as a dietary supplement which is rich in proteins. Here the supplement consists of dried cyanobacteria [34].

Violacein: It is a versatile pigment from a bacterium *Chromobacterium violaceum* that exhibits numerous biological activities. It has gained immense importance in industrial markets, such as in medicine, cosmetics, food and textiles [35].

Astaxanthin: Chemically it is 3, 3'-dihydroxy-b, b-carotene-4, 4'- dione and is an orange - red pigment and produced by microorganisms such as red Basidiomycetous yeast *Xanthophyllomyces dendrorhous* [36], green algae *Heamatococcus pluvialis* and *Agrobacterium aurantiacum* [33].

Pigment compounds form microorganisms based on color and appearance can be classified as shown in Table 2.1.

Table 2.1.Pigment producing microorganisms based on color and appearance.

Microorganism(s)	**Pigments/Molecule**	**Color/appearance**	**Reference**
Bacteria			
Agrobacterium aurantiacum	Astaxanthin	Pink-red	[19]
Paracoccus carotinifaciens	Astaxanthin	Pink-red	[15]
Bradyrhizobium sp.	Canthaxanthin	Dark- red	[15]
Flavobacterium sp., *Paracoccus zeaxanthinifaciens*	Zeaxanthin	yellow	[37-38]
Achromobacter sp.		Red	[39]
Brevibacterium sp.	Carotenoid like	Orange yellow	[40]
Corynebacterium michigannise		Greyish to creamish	
Corynebacterium insidiosum	Indigoidine	Blue	[41]
Rugamonas rubra, Streptoverticillium rubrireticuli, Vibrio gaogenes, Alteromonas rubra	Prodigiosin	Red	[22]
Xanthophyllomyces dendrorhous	Astaxanthin	Pink	[42]
Haloferax alexandrinus	Canthaxanthin	Dark Red	[43]
Staphylococcus aureus	Staphyloxanthin Zeaxanthin	Golden Yellow	[44]
Chromobacterium violaceum	Violacein	Purple	[45]
Serratia marcescens, Serratia rubidaea,	Prodigiosin	Red	[46]
Pseudomonas aeruginosa	Pyocyanin	Blue-green	[47]
Xanthomonas oryzae	Xanthomonadin	Yellow	[48]
Janthinobacterium lividum	Violacein	Purple	[49]
Algae			
Dunaliella salina	β-carotene	Red	[50]

Chlorococcum sp.	Lutein	Orange	[51]
Hematococcus sp.	Canthaxanthin	Yellow-orange-red	[52]
Fungi			
Aspergillus sp.	β -carotene	Orange-red	[53]
Blakeslea trispora	β -carotene	Cream	[54]
Fusarium sporotrichioides	Lycopene	Red	[55]
Haematococcus Pluvialis	Astaxanthin	Red	[56]
Monascus sp.	Monascorubramin Rubropunctatin	Red - orange	[57]
Monascus purpureus	Monascin Ankaflavin	Red-yellow	[22]
Monascus roseus	Canthaxanthin	Orange-pink	[22]
Monascus sp.	Ankaflavin	Yellow	[22]
Penicillium oxalicum	Anthraquinone	Red	[58]
Blakeslea trispora	Lycopene	Red	[59]
Cordyceps unilateralis	Naphtoquinone	Deep blood-red	[60]
Mucor circinelloides, Neurospora crassa and Phycomyces blakesleeanus	β-carotene	Yellow-orange	[61]
Pacilomyces farinosus	Anthraquinone	Red	[62]
Yeast			
Cryptococus sp.	Melanin type compounds	Reddish brown	[63]
Saccharomyces neoformans var. *nigricans*	Melanin	Black	[19]
Phaffia rhodozyma	Astaxanthin	Pink-red	[64]
Rhodotorula sp. *Rhodotorula glutinis*	Torularhodin	Orange-red	[65]
Yarrowia lipolytica	Melanin	Brown	[66]
Actinomycetes			
Streptoverticillium rubrireticuli	Prodigiosin	Red	[67]
Streptomyces echinoruber	Rubrolone	Red	[68]

2.7 Microbial pigments of commercial importance

The success of any microbial pigment produced by biotechnological means (for example fermentation) depends upon its acceptability in the market, regulatory approval, and the size of the capital investment required in bringing the product to the market. A few years ago, some expressed doubts about the positive commercialization of fermentation derived food grade pigments because of the high capital investments requirements for fermentation facilities and the expensive and time-span toxicity studies required by regulatory agencies [15, 19].

In addition to the above public perception of biotechnology derived products should also be taken into account for the absolute production benefit. Table 2.2 states the successful microbial pigments already in use as food grade and/or nutritional supplements in the market and are derived from various bacteria, yeast and fungi. Based on the extensive research reports, these pigments broadly can be categorized into pigments of industrial production (IP), developmental stage (DS) and research project (RP) phase (Table 2.2) [19, 22].

Microbial colorants are in use in the fish industry already, for instance, to enhance the pink color of farmed salmon. Further, some natural food colorants have commercial prospective for use as antioxidants [69]. Nowadays several fermentative food grade pigments are in the market: *Monascus* pigments, *astaxanthin* from *Xanthophyllomyces dendrorhous*, Arpink Red from *Penicillium oxalicum*, riboflavin from *Ashbyagossypii*, and carotene from *Blakeslea trisporatrispora* [15, 19, 24] which are considered harmless and approved by FDA. The successful marketing of pigments derived from microbes, both as a food color and a nutritional supplement, reflects the presence and significance of niche markets in which consumers are willing to pay a premium for ‘all natural ingredients’.

Table 2.2. Pigments from various microorganisms which are already in use as natural food colorants [19, 22].

Pigment	Color	Microorganism	Status
Ankaflavin	**Yellow**	***Monascus sp.***	**IP**
Anthroquinone	**Red**	***Pencilllium candidum***	**IP**
Monascorubramine	**Red**	***Monascus sp.***	**IP**
Riboflavin	**Yellow**	***Ashbya gossypi***	**IP**
Rubropanctatin	**Orange**	***Monascus sp.***	**IP**
β Carotene	**Yellow-orange**	***Blakeslea trisporia***	**IP**
Astaxanthin	**Pink-red**	***Agrobacterium aurantiacum***	**RP**
Astaxanthin	**Pink-red**	***Paracoccus carotinifaciens***	**RP**
Cathaxanthin	**Dark red**	***Bradirhizobium sp.***	**RP**
Lycopene	**Red**	***Fusarium sporotrichioides***	**RP**
Melanin	**Black**	***Saccharomyces neoformis***	**RP**
Napthoquinone	**Deep blood red**	***Cardyceps unilateralis***	**RP**
Zeaxanthin	**Yellow**	***Paracoccus zeaxanthinifaciens***	**RP**
β Carotene	**Yellow-orange**	***Fusarium sporotrichioides***	**RP**
β Carotene	**Yellow-orange**	***Neurospora crassa***	**RP**
β Carotene	**Yellow-orange**	***Phycomyces blaksleeanus***	**RP**
Unknown	**Red**	***Paecilomyces sinclairii***	**RP**
Astaxanthin	**Pink-red**	***Xanthophyllomyces dendrohous***	**DS**

Lycopene	**Red**	***Blakeslea trisporia***	**DS**
Rubrolone	**Red**	***Streptomyces echinoruber***	**DS**
Torularhodin	**Orange-red**	***Rhodotorula sp.***	**DS**
Zeaxanthin	**Yellow**	***Flavobacterium sp.***	**DS**
β Carotene	**Yellow-orange**	***Mucor circinelloides***	**DS**
Unknown	**Red**	***Penicillium purpurogenum***	**DS**

The number of approved colorants for food industry is restricted. Some approved food colorants are recognized by their chemical name (for eg. canthaxanthin) while others are known by source (eg. fruit juice or vegetable juice). The biocolorants identified by their chemical name can be synthesized easily by cheaper biotechnological sources particularly by various microorganisms. And technological limitations are the major hold-up for the commercial exploitation of the source materials [15, 61]. The success of any pigment produced by fermentation rely up on its acceptability in the market, regulatory approval, and the size of the capital investment required in bringing the product to market [61]. Some food grade pigments by microorganisms with commercial value are given in Fig. 2.1.

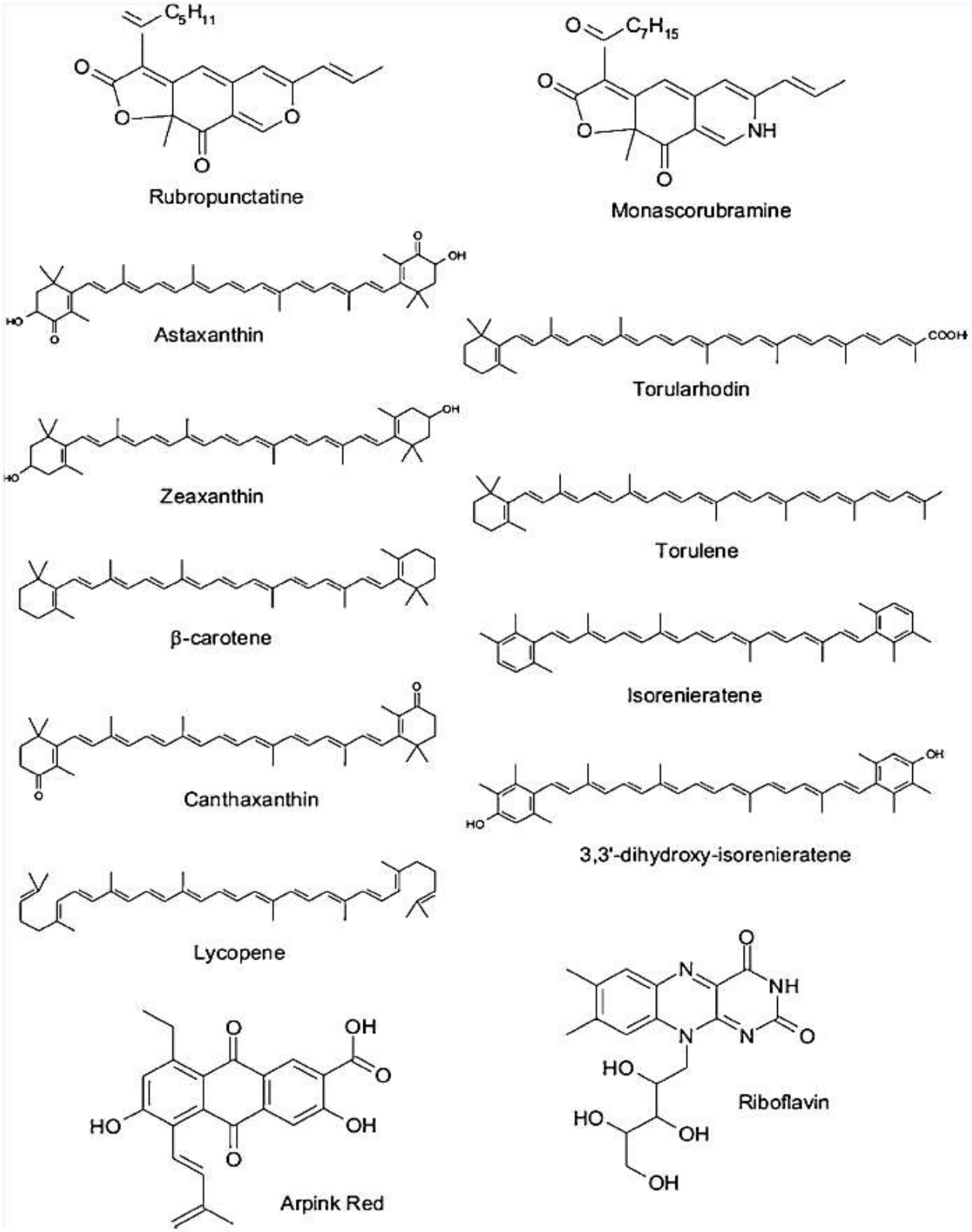

Fig. 2.1. Some food grade pigments and their structures from microorganisms [15].

2.8 Benefits and Applications of Microbial pigments

Pigments produced by microorganisms are of traditional use in oriental countries and have been a subject of intense research in the present decades because of its potential for applications. Use of microorganisms and biotechnology would offer solutions to the problems of various industries especially food colorant industry. Fermentative production of colorants has a number of benefits that include: cheaper production, readily available raw materials, high yields and no seasonal variations [70].

In contrast to higher plants, single cell algae and fungi are more appropriate for biotechnological production because they can be grown using existing culture techniques. Many fungi produce pigments which have application in both textile and food industries [71]. Fungal pigments are routinely utilized as colorants for both foodstuffs and materials. Some of the more importantly utilized fungal pigments come from the water soluble orange/red pigments produced by *Monascus* sp., frequently used in rice wines in eastern countries [72]. Pigments produced from *Monascus purpureus* Piedallu are used in wool dying [73], and an anthraquinone pigment obtained from *Penicillium oxalicum* Currie & Thom is currently being developed for use as a 'natural' food additive that may have some anticancer effects [14, 16]. Mycelial extracts of some promising mushrooms are *Chroogomplus vinicolor* (which gives red tints), *Bankera violascens* (which gives greens) and *Collybia iocephala* (which gives blues), they have a remarkable potential for dyeing wool and silk fabrics [74]. Carotenoids such as β-carotene and lycopene have been known to be produced by fungal cell factories. For example, β-carotene by *Blakeslea trispora*, *Mucor circinelloides*, and *Phycomyces blakesleeanus* and lycopene from *Fusarium sporotrichioides, Blakeslea trispora* are already in use as for food colorants [33]. Vitamin, riboflavin (vitamin B2) is a yellow

food colorant that is fermentatively produced by the fungi *Eremothecium ashbyii* and *Ashbya gossypi* and has huge commercial potential [75].

Microalgae such as *Haematococcus* (astaxanthin), *Spirulina* (phycocyanin), and *Dunaliella* (ß-carotene and other carotenoids) are already explored as sources of natural food colorants with some limitations of low productivity and contamination in the open culture system, where they are grown [76]. Newly, a blue-green polyphenolic antioxidant pigment termed marennine from the Diatom *Haslea ostrearia* has been reported [77]. This may aid as a promising food coloring additive.

Among yeasts, the marketable success has been with the case of astaxanthin production by *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) and is widely in use as food additive. Additional example of production of carotenoids is by the yeast *Rhodotorula* spp., *R. glutinis, R. gracilis, R. rubra, and R. graminis.* The chief carotenoids produced by these yeasts are torulene and torularhodin, with minute quantity of β-carotene [78-81].

The pigments obtained from bacteria offer the following benefits and advantages [15, 24]; they are progressively attractive to science because of broad ranging activities, easy propagation and wide-ranging strain selection, high versatile and productive compared to other sources, fermentation is integrally faster and more productive production when compared to any other chemical process, simple and fast culturing methods allowing continuous bioreactor operation, quite easy to manipulate genes, structural complexity suits for industrial requirements, Cheap substrates used for bulk production and their pigments extracted using simple liquid-liquid extraction technique minimizing operation cost. Some important functions of bacterial pigments of commercial potential are 1) Riboflavin

synthesized by *Bacillus subtilis* and used in foods, vitamin enriched milk products and energy drinks, 2) β-carotene usually produced by *Flavobacterium, Agrobacterium aurantiacum* and widely used as food supplement for humans and as food additives for animals and fish, 3) Prodigiosin by *Serratia marcescens*, *Pseudomonas*, *Pseudoalteromonas*, *Alteromonas denitrificans*, *Hahella*, *Vibrio* species used as antibacterial, antifungal, antimalarial, antibiotic, immunosuppressive, anticancer etc., 4) Phycocyanin by *Cyanobacteria* used as dietary supplement rich in proteins, and 5) Violacein by *Chromobacterium violaceum,Alteromonas luteoviolacea* was used as Antiviral, antibacterial, antiulcerogenic, antileishmanial, and anticancer properties, potent cytotoxic effects against U937 and HL60 Leukemia cell lines [15, 24].

2.9 Novel practices of microbial pigment production

2.9.1 Strain improvement

Traditionally, strain development was achieved mainly by multiple rounds of random mutagenesis and selection, which are still very helpful nowadays. In the latest decade, the development of gene deletion techniques enabled efficient genome DNA inactivation and greatly improved metabolic engineering of bacteria [15, 82-83]. A more systematic and integrated approach for biotechnological process for strain improvement became prevalent. The motivation for industrial strain development is economic, since pigment amounts produced by wild strains are too low for cost-effective process. It is very essential to isolate strains which produce pigments with shorter fermentation times. Improvement of microbial strains for the over production of industrial products has been the characteristic of all commercial fermentation processes. The strain improvement by common mutagens such as

ultraviolet (UV), ethyl methane sulfonate (EMS) and 1-methyl-3-nitro-1-nitrosoguanidine (NTG) is convenient and can improve pigments yield as proved in several cases [15, 84]. New methods like X-ray and fast neutron of irradiations are also being tried to achieve microbial strain improvements. For instance, *M.purpureus* was subjected to the above irradiations to bring about change in pigmentation and morphology [25].

2.9.2 Fermentation

Fermentation is a metabolic process that converts sugar to acids, gases and/or alcohol and usually occurs in yeast and bacteria. The production of microbial pigments by fermentation is an interesting area and a lot of attention is now paid to this biotechnological approach. This technique can be of solid or submerged fermentation mainly and each approach is having its own merits and demerits in pigment production.

Submerged fermentation

Submerged fermentation utilizes free flowing liquid substrates, such as molasses and broths. The bioactive molecules are secreted into the fermentation broth. The substrates are utilized rapidly; hence need to be constantly replaced or supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An added advantage of this technique is that purification of products is easier [85]. For example *Monascus* has been successfully cultured submerged condition for pigment production and versatile substrates like breads, rice and other amylaceous (starch, dextrins, glucose, maltose and fructose) materials for high productivity of red pigments which occurs due to glucose and maltose utilization [85]. Carotenoid production by *Aspergillus* sp.[86] melanin type pigment by *Aspergillus niger* [87], water soluble red pigment by *Penicillium purpurogenum* [88]are some reported studies from fungi. Furthermore **m**any

bacterial pigments too successfully produced using this technique [15]. Various commercial scale microbial pigments such as prodigiosin, monascorubramie, astaxanthin, canthaxanthin, beta-carotene and so on are produced using this method. Under submerged conditions, factors like nitrogen, pH temperature majorly affect the pigment production [15, 25, 33].

Solid- state fermentation

Solid state fermentation (SSF) is defined as any fermentation process performed on a non-soluble substance that acts both as physical support and source of nutrients in absence of free flowing liquid [89]. SSF utilizes solid numerous versatile substrates, like bran, bagasse, and paper pulp etc. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates [89-90]. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high aw (water activity), such as bacteria [91]. Many pigments were reported using this technique [92-93] and among all *Monascus* pigments lead with many reports [94-96]. For example red rice, a traditional Asian food, has for thousands of years been prepared by fermentation of steamed rice by *Monascus purpureus* in SSF. The fungus produces six different polyketide pigments colored from bright yellow to deep red, which have found applications both as food additives and pharmaceuticals [25, 97]. For significant amounts of pigments by molds, substrates like rice, cassava or corn, wheat, oat and barley were used as substrates [93]. *Aspergillus* sp. has been grown successfully on starch medium using different carbon sources wherein dextrin and maltose produced the highest color intensity [25]. Some

significant studies include carotenoid by *Penicillium* sp. [98], riboflavin, a yellow pigment from *Eremothecium ashbyii* and *Ashbya gossypi*, beta-carotene by *Dunaliella* and *D. bardawil*, astaxanthin by *Haematococcus pluvialis*, phycocianin by *Spirulina* sp. [92, 99] etc.

2.9.3 Low-cost substrates

Agro-industrial by-products and surpluses that frequently create serious environmental problems may be possibly used as inexpensive carbohydrate sources for microbial fermentations, thus decreasing their initial high biological oxygen demand (BOD) while obtaining biochemical compounds like pigments suitable for pharmaceutical, chemical and food industries [100]. Low cost by products and residues of agro-industrial origin have shown their potential in production of different pigments by diverse group microorganisms. Along this line, variety of substrates and microorganisms has been tested. beta-carotene synthesis by citrus products [101], carotenoids production using whey ultrafiltrate [102], sauerkraut brine [81] and peat extract [103], riboflavin in concentrated rectified grape must [104], astaxanthin on grape juice [105] are some promising studies.

These by-products from industrial processes and other agro or domestic sources are pollutants to the environmental and their treatment involves high costs. The conversion of these wastes to value added materials like pigments by microorganisms would provide economic benefits and reduce waste materials impact on environment as pollutants. Many investigations have been performed and are under investigation to diminish the costs and optimize the pigments production [78, 86, 106-111]. Factors such as carbon and nitrogen source are very important to consider on the selection of wastes as substrates. And pigment production also depends on minerals and other components in some cases. Pigments produced on several wastes were shown in Table 2.3.

Table 2.3. Different microorganisms and various inexpensive substrates used for pigments production.

Substrate	**Microorganism**	**Pigment type**	**References**
Whey	*R. glutinis*	β -carotene	[112]
Potato medium	*R. mucilaginosa*	β -carotene	[112]
Crude glycerol	*R. glutinis*	carotenoids	[113]
Chicken feathers	*R. glutinis*	carotenoids	[114]
Fermented radish brine	*R. glutinis*	β -carotene	[115]
Mung bean waste flour and sweet potato extract	*R. glutinis*	carotenoids	[79]
Mustard waste	*X. dendrorhous*	Astaxanthin	[100]
Plant extracts	*X. dendrorhous*	Astaxanthin	[116]
Coconut milk	*X. dendrorhous*	Astaxanthin	[117]
Enzymatic hydrolysates of prehydrolysed wood	*X. dendrorhous*	Astaxanthin	[118]
Sugarcane waste	marine *Streptomyces* sp.	melanin	[119]

2.10 Objectives of the work

The overall objectives of this study are to explore the possibility of pigment production by different microbial isolates from numerous sources on various substrates. Furthermore, the enhanced pigment production capability of carotenoid producing microorganisms was also investigated using fruit waste material as sole substrates. The influential parameters of increased pigment production by several optimization techniques were also investigated.

The specific objectives of this study are:

- Isolation, identification and characterization of the microorganisms from different sources responsible for various pigment production
- Investigate and determine effects of substrate (cost-effective), pH, temperature, moisture, agitation, etc., on the pigment production process by respective microorganism
- Optimize the key parameters for improved pigment production and validation of the established procedure
- Exploring the potential of the obtained pigments as antioxidants, metal chelating agents, photoprotective agents etc.
- Investigating known microbial strains towards enhanced pigment production on various agro-industrial substrates

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Chapter 3

Screening of inexpensive substrates for pigments production by isolated and known microbial strains - Bridge to Chapter 3 to 8

3.1 Melanins and Carotenoids - pigments of marketable importance

Owing to a growing worldwide market for pigment compounds by microbial production, carotenoids and melanin compounds has great potential in terms of applications and strategies of production. Among both, carotenoids occur as natural colorants with a range of yellow to red colors, so they have great influence on the acceptability of many foods [1-2]. Moreover, some carotenoids are precursors of vitamin A; in terms of human health, they are amongst the bioactive phytochemicals credited that reduce risks for degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract [3-4]. Carotenoids are naturally occurring lipid-soluble pigments, the majority being C_{40} terpenoids, which act as membrane protective antioxidants scavenging O_2 and peroxyl radicals; their antioxidant ability is

apparently credited to their structure. Carotenoids pigments occur commonly in photosynthetic systems of higher plants, algae and phototrophic bacteria. On the other hand, in non-photosynthetic organisms, carotenoids are significant in protecting against photo-oxidative damage. Thus, many non-phototrophic bacteria and fungi rely on carotenoids for protection when growing on conditions where light and air are copious [3-7]. The carotenoids production by the organisms follow two distinct pathways, the well-known mevalonate (MVA) pathway and the relatively new 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. This pathway produce isopentenyl diphosphate (IPP) and its isomer of dimethylallyl diphosphate (DMAPP), the universal building blocks for the synthesis of all carotenoids. Eukaryotes generally use the MVA pathway to convert acetyl–CoA to IPP, which is subsequently isomerized to DMAPP. Prokaryotes, with some exceptions, use the MEP pathway to produce IPP and DMAPP via an initial condensation reaction between pyruvate and glyceraldehyde-3-phosphate (G3P). Plants and *Streptomycetes* follow both pathways for the synthesis of these commercially significant pigments [8-9].

Further coming to melanin compounds, these are interesting materials that are currently being used in diverse fields such as medicine, pharmacology, cosmetics etc. In addition, many different technological applications including photo-voltaics, sensors, electronic devices and may prove to be a novel class of semiconducting polymers, bio-compatibles and with a readily available natural source as natural materials extracted from pigmented [10-11]. However, despite momentous scientific effort over the past 30 years, the basic functions of melanins are still a matter of controversy and speculation. This uncertainty imparts from the few and poorly defined structural and physical–chemical properties that are known in these molecules that continue to mystify researchers even though their adaptive

importance has already been proven [10, 12]. These materials are predominantly intractable from an analytical perspective because they are chemically and photo-chemically very stable, and they are virtually insoluble in most common solvents. In microorganisms, melanins may be found in both extracellular or intracellular environments, and they pretence very interesting targets for molecular structure determination and organic synthesis [13-16]. Melanins constitute a general class of complex, polyphenolic heteropolymers and are usually divided into brown–black melanins (called as eumelanins) and brown, red or yellow melanins (called as pheomelanins). A number of bacterial species have been testified to produce melanins [11, 17]. These compounds can be produced in the *Actinomiceto* or *Streptomicetos* groups, *Bacillus* and *Azotobacter* genuses [11, 18]; however, very few of these pigments have been molecularly defined. The well-known melanisation pathway is the classic Mason–Raper pathway. In this pathway, tyrosinases yield the melanin intermediate dihydroxyphenyl-alanine (DOPA), dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA) [10-11, 19]. They are some other melanin types and other synthesis pathways are also reported that produce melanins; for example, pyomelanin is derived from the catabolism of tyrosine via *p* -hydroxyphenylpyruvate and homogentisic acid (HGA) [20].

First and foremost, production of melanin is one of the most universal adaptations of living organisms to the variable conditions of the earth and these pigments. As these pigments are widespread among microorganisms, extensive research focused on exploration around this ubiquitous pigment which of commercial interest and have great application potentials in the agriculture, cosmetics and pharmaceutical industries. Additionally, the widespread synthesis calibre of catotenoids by heterotrophic and non-phototropic bacteria, fungi and yeast suggests that these pigments are crucial for the viability of these organisms in

their natural environment. Carotenoids are the largest and most diverse class of natural pigments known to mankind and they have enormous commercial significance with promising applications from food to textiles. The first part of our investigation engaged in isolation of individual melanin and carotenoid producing microorganisms which are majorly at RP and DS of research. Here microbial cultures were collected from various natural resources such as marine and soil samples The second part of our work involves the employment of the selective pigment producing microbial isolates on various low-cost by-products and solids as substrates for effective pigment (caroteniod or melanin) production. This chapter ends by extensive screening of the collected inexpensive wastes as sole substrates for pigment production by the purchased microbial strains.

3.2 Sampling

Water

Marine water samples were collected from different sites along the coast of Vishakhapatnam beach, Andhra Pradesh, India. Sampling locations were shown in Table 3.1.

Soil

Soil sample was collected from the garden of NIT campus Rourkela. The obtained soil was thoroughly washed with sterile distilled water to separate the floating dust and debris.

The soil suspension and the collected water samples were then aseptically diluted serially (10^{-7} and 10^{-10} times where ever necessary) and 1ml of it was inoculated on various mediums and observed for various diffusible pigments and/or pigmented colonies. All the isolates were cultivated at ambient conditions i.e. at pH 7 and temperature 30 ^{0}C. For liquid media an agitation of 150 rpm was maintained throughout the incubation process.

3.3 Screening of pigment producing microorganisms

Isolates from different sources were cultured on their respective growth media and at specified growth conditions; these were awaited for 2-10 days for visible colored colonies. Primarily, after incubation for two days, marine isolate from deep sea water gave creamy and purple colonies; while isolates from shore (near rocks) gave yellow and black colonies. The observed colonies were picked and cultured separately for individual pure cultures. Coming to soil isolate; nutrient agar was used as culture medium. Here various colonies were seen after 2 days, whereas orange pigmented colonies were observed after 10 days of incubation. These colored colonies were picked and sub-cultured for further experimentation.

3.4 Different substrates and Cultivation of isolates/purchased strains

In this work, cabbage (*Brassica oleracea*) waste was prepared from the outer leaves of cabbage that are peeled off before cabbages are distributed in the market. And potato peels and jack fruit seeds were collected from domestic disposed wastes. The obtained raw wastes were dried in oven at 60 ^{0}C for 12 hrs, powdered and used as substrates for screening microbial isolates for pigment production. A 5 % solution was prepared from the respective powders and used as liquid medium for the cultivation of isolates.

Free water or liquid medium does not appear to be natural habitat for the majority of the microorganisms. Solid substrates in this case enable favorable conditions (close to the natural environment to which microorganisms are adapted) of growth especially for wild type microorganisms. Therefore solid substrates such as rice, corn and wheat flour powders (obtained from local market) were explored by the isolated microorganisms for pigment

production. Substrate medium was prepared by autoclaving (at 15 lb pressure for 10 min) 3 g of individual powders in a petridish (10 cm) with 8 mL distilled water.

Another waste used in the work was from fruit wastes. Fruit waste was obtained from a fruit juice shop of a local market. The material used is from single batch i.e. used in all the experiments to minimise the disturbances in the results due to variations in composition. The waste contains major portion of pine apple and orange waste and minor portions of pomegranate waste. Fruit waste includes extracted carpels of oranges, core of pineapples, and crushed seeds along with arils of pomegranate. The soluble sugars are extracted from 1 kilogram of fruit waste by adding 2 litres of distilled water and boiled at 100 ^{0}C for 30 minutes. The resultant straw colour fruit waste extract (FWE) was filtered and stored at 4 ^{0}C for further experimentation.

The prepared solid and liquid media are used for pigment production studies by marine and soil microbial isolates. These were incubated for 6-7 days at 25 °C and REMI shaking incubator (operated at 150 rpm) was used for liquid medium substrates. Carotenooid producing strains *Rhodotorula rubra*. (MTCC no: 1446) *Xanthophyllomyces dendrorhous* (MTCC No: 7536) were obtained from imtech Chandigarh and were cultivated on above specified wastes for pigment production.

3.5 Strain/substrate selection and further experimentation

Selection of the appropriate substrates - for microbial isolates

The pigment compounds presences in various wastes along with selective media by microbial isolates are shown in Table 3.1. Upon exploitation, marine isolate taken from near shore area

resulted in black color which is moderate in vegetable waste medium as substrate when compared to significant color change in marine agar.

Furthermore two microbial strains isolated from garden soil; independently resulted in black color when cultivated on fruit waste extract medium, and an orange color in solid rice powder medium when used as solitary substrates. Rice powder medium was significantly colored in orange than the defined nutrient agar medium by garden soil isolate.

The mediums devoid of color change were discarded and omitted from the study. Vegetable waste from cabbage, fruit waste extract and rice powder are therefore selected for additional investigations.

Selection of the appropriate substrates - for carotenoid producing microorganisms

Like microbial isolates, various growth mediums were tested by the purchased microorganisms (yeast) which are potent carotenoid producers on defined and undefined mediums. Color change of the media was observed for the selection of the best substrate for pigment production by the obtained microbes (Table 3.2).

Among all the mediums, fruit waste extract medium significantly changed its color when cultivated by respective yeast. *R. rubra* produced vibrant pink-orange color, while *X. dendrorhous* gave intense orange-red to the medium comparable to the standard nutrient medium. This FWE medium was subjected to further investigation using these yeasts.

Table 3.1. Screening of various substrates by marine water and soil isolates for pigments production. ++, + - indicate qualitative significance of high and moderate pigment production

Sampling location		**Color/pigmented colonies observed**	**Marine agar**	**Nutrient agar**	**FWE**	**Vegetable waste**		**Jack fruit seed waste**	**Rice powder**	**Corn flour**	**Wheat flour**
						Cabbage waste	**Potato peels**				
Marine	Deep sea water	**Creamy, purple**	++	-	-	-	-	-	-	-	-
	Shore	**Black**	++	-	-	+	-	-	-	-	-
		Yellow	++	-	-	-	-	-	-	-	-
Soil	Garden	**Black**		++	++	-	-	-	-	-	-
		Orange		+	-	-	-	-	++	-	-

Table 3.2. Screening of various substrates for pigments production by purchased strains. ++, + - indicate qualitative significance of high and moderate pigment production

Species	**Nutrient medium**	**FWE**	**Vegetable waste**	**Potato peels**	**Jack fruit seed powder**	**Rice powder**	**Corn flour**	**Wheat flour**
Rhodotorula rubra. (MTCC no: 1446)	++	++	-	-	-	-	-	-
Xanthophyllomyces dendrorhous (MTCC No: 7536)	++	++	-	-	-	-	-	-

3.6 Summary

Use of microbial pigments in processed foods, cosmetics and in other applications is promising with large economic potential. However, microbial pigments present several challenges due to high cost, lower stability and disparity in shades due to changes in pH.

At present, none of the microbial pigment can replace synthetic pigments. But recent advances and efforts in synthetic biology, metabolic engineering etc. will greatly expand the pigments that could be produced economically in sufficient amounts for industrial application.

Extensive efforts on fermentation process, utilization of low-cost by-products and residues of agro-industrial origin as alternative substrates have shown the possibility of attaining non-toxic microbial pigments in significant amounts. Research in this area is escalating as the outputs achieved here are fast, have economic potential with less efforts and these technologies can be implemented immediately than the time taking genetic engineering of microorganisms. Utilization of wastes, screening of novel microorganisms for non-toxic pigments, optimization of production conditions are our major objectives of focus in the coming chapters of the work.

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Chapter 4

Approach and investigations

Part I - Isolation of Pigment Producing Bacteria and the Production of Pigments on Cheaper Substrates

4.1 Melanin from Isolated Marine *Pseudomonas* sp. using Vegetable waste

4.1.1 Introduction

Melanins are diverse group of macromolecules, synthesized ubiquitously in living organisms by oxidative polymerization of various phenolic substances in the process of adaption [1]. In nature, melanins act as photoprotectants (against UV and visible light), charge transport mediators, free-radical scavengers, antioxidants, metal ion balancers and etc. [2]. Melanins also have applications in agriculture, medicine, cosmetic and pharmaceutical industries. In general, melanins are negatively charged, hydrophobic, high molecular weight compounds with amorphous nature. These are insoluble in common organic solvents, aqueous acids and water [1],[3].

Based on color and structural classes primarily there are three types of melanins i.e. eumelanins, pheomelanins and allomelanins. Eumelanins are black to brown color pigments produced by melanisation by classic Mason-Raper pathway, which produce tyrosine intermediates or metabolites by the action of tyrosinases. Pheomelanins are brown, red or yellow color pigments which are produced in course of oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DOPA) and dopaquinone. Pheomelanin results from cysteinylation of DOPA and these are sulphur containing compounds. Allomelanins include nitrogen free heterogeneous group of polymers formed from catechol precursors [3],[4]. The eumelanins and pheomelanins commonly occur in animal species, while allomelanins can be seen in microorganisms and plants [5]. Some of the funguses known to produce melanins are *Cryptococcus neoformans*, *Sporothrix schenckii*, *Sepia officinalis*, *Aspergillus niger*, *Penicillium marneffei*, *Paracoccidioides brasiliensis*, *Histoplasmacapsulatum*, *C. neoforman* [6]. some bacterial species include *Aeromonas salmonicida*, *Azotobacter*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Legionella*, *Streptomyces*,

Rhizobium, *Vibrio*, *Proteus*, *Azospirillum*, *Pseudomonas aeruginosa*, *Hypomonas* sp, *Burkholderia cepacia*, *E. coli*, *Bordetella pertusis*, *Campylobacter jejuni*, *Yersinia pestis* etc. [2],[5].

Apart from terrestrial microorganisms, explorations of melanin production by marine microorganisms appear to be inadequate due to limited literature. For instance, Kotob et al [7] synthesized marine melanin from *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. They reported that the formed melanin was pyomelanin that resulted due to catabolism of tyrosine via Tyrosine degradation pathway. Another study by marine bacterium genus *Alteromonas* produced melanin in-vivo with the aid of tyrosine precursors [8]. Similarly, other marine bacteria capable of producing melanin are *Marinomonas mediterranea* MMB-1T which belong to the phylum *Proteobacteria* [9] and thermo-alkaliphilic *Streptomyces* (from limestone quarries of the Deccan traps) [10]. Apart from bacteria, a study by obligate marine fungus *Cirrenalia pygmea* showed melanin production ability in its mycelium and conidia [11].

Overall, melanin pigment from various microbial species especially from marine species is an attractive option of research still in its infancy. In this study, we describe a procedure for the isolation of melanin producing marine microorganism (*Pseudomonas* sp.) and characterized biochemically. Melanin producing ability of the isolate was further tested on vegetable waste (pure and blended with marine broth) at ambient temperature and pH. The structure of the bacterium and pigment nature was identified by scanning electron microscopy and the synthesized melanin was analyzed spectrophotometrically. The extracted and purified pigment was characterized using energy dispersive spectroscopy and Fourier infrared spectroscopy.

4.1.2 Materials and methods

4.1.2.1 Chemicals used

Marine agar, marine broth, DPPH (2,2-diphenyl-1-picrylhydrazyl) were procured from HiMedia chemicals, Mumbai, India. Ethanol, NaCl, NaOH, HCL and all other chemicals used were of analytical reagent grade throughout the study. Ultrapure water used for the experiments and aseptic conditions were maintained wherever necessary.

4.1.2.2 Screening and isolation of the melanin producing strain

Microorganisms capable of producing melanin were isolated from the sea water samples collected from three different locations i.e. nearby rocks (sample 1), shore (sample 2) and from deep sea water [10 m away from shore] (sample 3) of Vishakapatnam beach, Andhra Pradesh, India. 0.1 mL of diluted water samples of 10^{-7} dilution was individually spreaded on marine agar plates at pH 7.0. The media and the glassware were autoclaved at 15 psi (121 ^{0}C) for 20 min prior to the experiment. These agar plates with media and inoculum were incubated at 25 ^{0}C for 48 h. Melanin producing microorganisms were identified by the presence of microbial colonies with dominant thick black color (diffusible) in agar plates. Selective colonies were separated out for sub-culturing and characterization.

4.1.2.3 Pigment production, extraction and purification

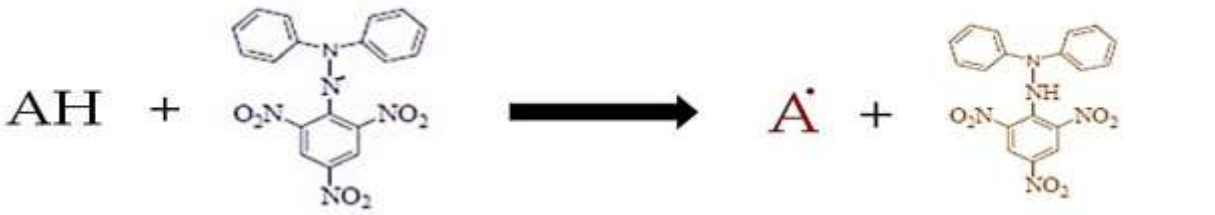
Marine broth medium was used for inoculum preparation and pigment production. About 10 µL (10^8 CFU/mL) culture suspension was added to 50 mL marine broth in 250 mL flasks. This medium was then incubated at 25 ^{0}C on a rotary shaker moving at 200 rpm for 48 to 72 h until the liquid medium was darkly pigmented and nearly opaque. All media used for the study were sterilized by autoclaving unless elsewhere stated. After a specific incubation time, the medium was centrifuged by a centrifuge (REMI-RM12C, India) at 8000 rpm for 15 min

to separate the broth (supernatant) and the cells. The solid pellet of cells was separated and suspended in distilled water. These cells were again centrifuged to collect the supernatant. Melanin was extracted from the overall supernatant by acidification with 3 N HCl at pH-2 and allowed to stand for 48 h initially at room temperature. This process was repeated for 3 more days until no further precipitation occurred. Then the suspension thus obtained was boiled for 5 min to prevent the formation of melanoidins [1]. As a final point, crude pigment pellet was collected after centrifugation at 4000 rpm for 15 min. In addition to marine broth medium, the pigment production ability of the isolate was tested by culturing them upon vegetable waste (from cabbage), blend of marine broth and vegetable waste (10:90, 20:80, 30:70) as a nutrient source. Culture conditions and the rest of the protocol maintained same as described above.

4.1.2.4 DPPH/SPF/metal chelating assay

DPPH assay

DPPH (1, 1diphenyl2picrylhydrazyl) accepts an electron to form a stable diamagnetic molecule. The methanolic solution of DPPH of violet color has got a strong UV absorbance at 517nm. The presence of a reducing agent in this methanolic solution pairs the odd electrons of DPPH radical and further the solution fade color stiochometrically and also the absorbance of the solution decreases at 517nm. The schematic representation of the process was shown below



AH : Antioxidant compound

DPPH : 1,1-diphenyl-2-picryhydrazyl

The radical scavenging activity by melanin pigment was investigated by modified method of Ju et al. [12]. Primarily 1 mL of (15-100 μg/mL) microbial melanin or standard (Ascorbic acid) was added to 2 mL of DPPH in ethanol. After 30 minutes incubation at 37 ^{o}C the absorbance was measured at 516 nm using UV-spectrophotometer. Corresponding blanks were also taken respectively. The experiment was performed in duplicate. The absorbance of DPPH as control was measured at 516 nm. Lower absorbance of the reaction mixture indicated higher radical scavenging activity. The scavenging effect (%) was measured using the following formula:

DPPH inhibition (%) =[(Control absorbance -test absorbance)/Control absorbance] X 100

In vitro sun protection factor

Sunburn, premature skin aging, skin cancers and suppression of the immune system are all linked to exposure of skin to UV light. The ultraviolet (UV) spectrum of between 200 nm and 400 nm is commonly divided into three regions: UV-A: 320-400 nm; UV-B: 280-320 nm; UV-C: 200-280 nm.

The highest energy region, UV-C, is absorbed wholly by ozone in the stratosphere. Among the total solar UV radiation reaching the earth's surface, 6% is in the UV-B region and 94% in the UV-A. The potential of UV radiation to cause skin damage increases exponentially with decreasing wavelength. UV light at 280 nm is 1000 times more damaging than light at 340 nm, thus, a sunscreen's ability to block UV-B is more important to prevent the negative effects of sun exposure.

The severe effects of UV-A and UV-B exposure are both short-lived and reversible. These effects include mostly sunburn (or erythema) and tanning (or pigment darkening). Largely sunscreen products contain ingredients that provide adequate protection only against UV-B rays. Even those labelled as broad-spectrum sunscreens may offer only partial protection against UV-A radiation. Sunscreens are products applied to the skin to protect against the harmful effects of the sun's UV rays. Sunscreens are usually grouped into two key categories, namely chemical absorbers and physical blockers. Chemical absorbers absorb high-intensity UV rays while physical blockers reflect or scatter them. Chemical absorber compounds include avobenzone, padimate O, octyl methoxycinnamate, octisalate, and octocrylene. Physical blocker compounds include titanium dioxide and zinc oxide. The international standard for quantifying the damaging effects of UV radiation on skin is the erythemal action spectrum and the Calculation of Sun Protection Factor (SPF) was performed according to Mansur equation [13] stated below.

As per the adapted method, spectra of melanin samples were collected over the spectral range 400-280 nm with 1 nm data point resolution on a UV-visible UV-3200 double beam spectrophotometer (LABINDIA analytical Instruments Pvt Ltd, India). The SPF values of

melanin from microbial isolate and purchased melanin were determined by Mansur mathematical equation as follows:

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where, SPF=Sunscreen protection factor; EE(λ)=Erythremal effect spectrum, I(λ)=Solar intensity spectrum; Abs(λ)=Absorbance of sunscreen product; CF=Correction factor (=36.2 for SNOW LOTUS® SPF 15 and 30) were taken from Huang et al [13].

Determination of metal chelating activity

The ferrozine-based colorimetric assay permits the quantitation of iron in the solution phases effectively. Ferrous and ferric iron were detected equally well by the assay and the accuracy was unaffected by other divalent metal cations. Usually Fe (II) ions form a magenta colored solution with ferrozine that yields a maximum absorbance at 562 nm. This reaction is used to quantitatively analyse Fe^{+2} (aq) in solution using a spectrophotometer. The complex is stable between pH 4 and 10 approximately. This method is applicable in ranges of concentrations between ~0.010 mg/L and ~ 3mg/L or between 2 and 10 ppm.

The chelation of ferrous ions by the melanin pigment was estimated by the method of Huang et al. [13]. Different concentrations of melanin were mixed with a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All the tests and

analyses were done in duplicate and averaged. The inhibition of the melanin pigment, metal chelating activity in percentage (%) was calculated by the following equation:

Metal chelating effect (%)=$[(A_0-A_1)/A_0]$ X 100 %

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of the sample of the melanin pigment or standards. The control used contains $FeCl_2$ and ferrozine.

4.1.2.5 Analytical methods

The morphology of the microorganism and the purified pigment was examined by scanning electron microscope (SEM) [JEOL JSM-6480LV]. The compositional pattern was determined by energy dispersive X-ray (EDX) spectroscopy which is coupled to SEM. UV-visible spectrum of the melanin was observed using UV-vis. spectrophotometer (UV-3600 Shimadzu). The FTIR analysis of pigment was carried out after mixing with KBr using FTIR spectrophotometer (Perkin Elmer, Model No.S2000, USA).

4.1.3 Results and Discussion

4.1.3.1 Strain selection and characterization of the microorganism

Inoculated sea water samples on marine agar plates were observed each day for melanin producing microorganism. At ambient conditions, during the end of second day (~ 48 h) visible black color colonies with diffused black color was evident in the agar plates inoculated with sea water sample 1 (Fig. 4.1.1). The other isolates devoid of black color were ignored for further study. Colonies form black color marine agar plates were transferred to new agar plates and allowed to grow for 2 days.

The visible tiny colonies and morphology of the isolated microorganisms observed by SEM images (Fig. 4.1.2a and b) states that the microbe might be of bacterial origin. The microbe size range was 1.2 to 1.7 µm and was rod shaped in appearance. For detail evidence regarding black pigment producing organism, isolated strain was sent for microbial phenotypic characterization to Institute of microbial technology (IMTECH), Chandigarh, India. The microorganism was identified as *Pseudomonas* sp. and is closely related to *Pseudomonas guinea*. Table 4.1.1 list out the key morphological and biochemical characteristics of the isolated bacterial strain.

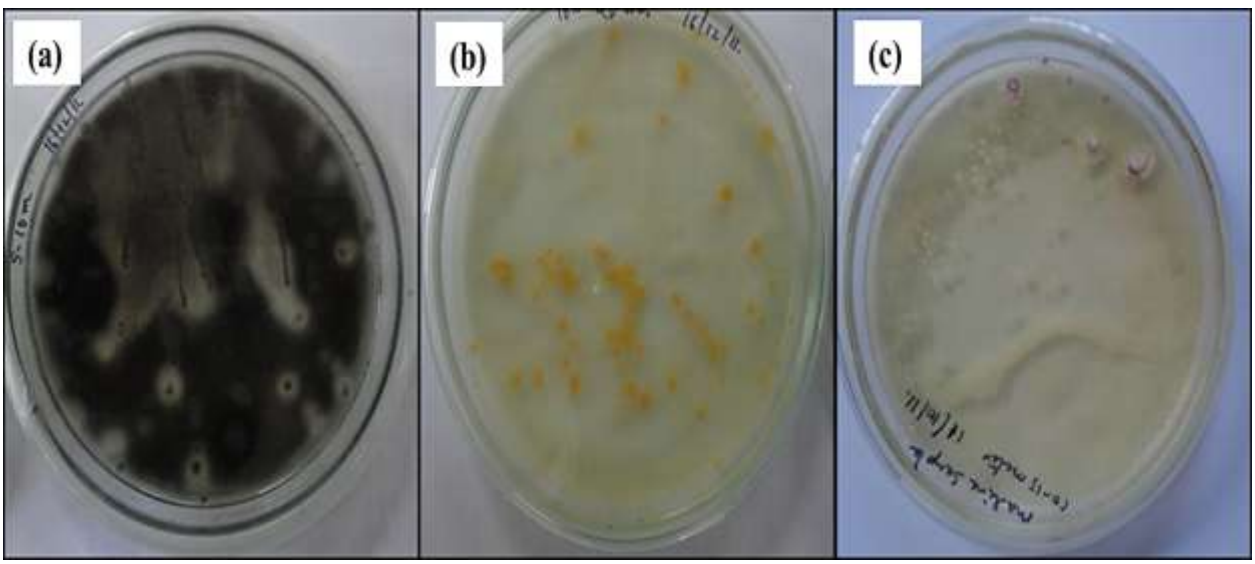


Fig. 4.1.1. Screening of microbial strains obtained from various parts of a sea shore. A) near stones, B) near shore and C) 10 m away from sea.

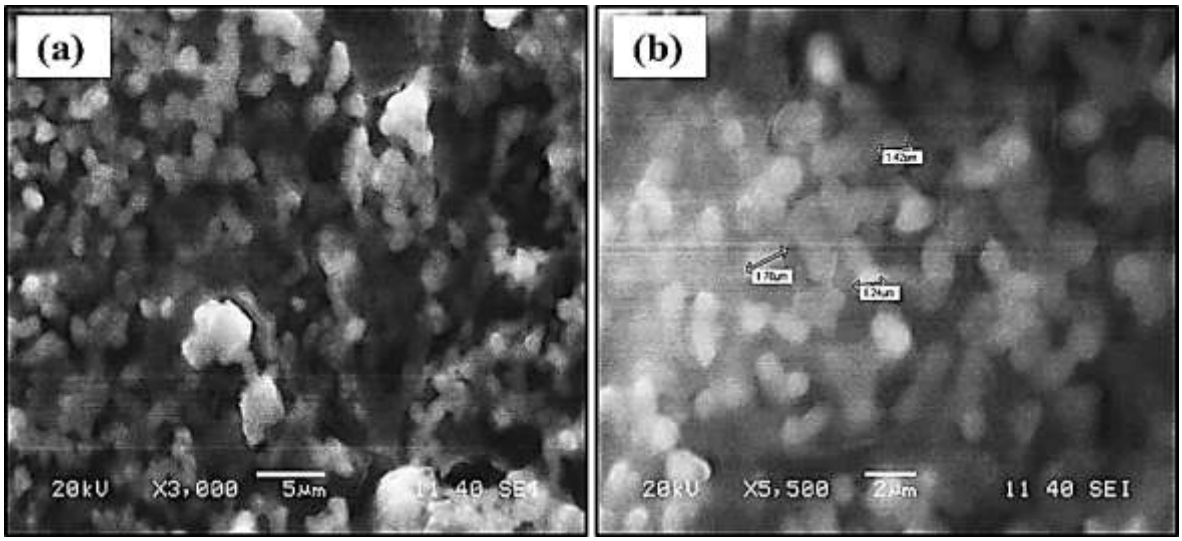


Fig. 4.1.2**.** Low (a) and high (b) magnification SEM images of the isolated microorganism with black colonies on agar plates.

Table 4.1.1. Colony characteristics of the isolated melanin producing bacterium.

Characteristics	**Result**
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Raised
Texture	Slimy
Opacity	Opaque
Gram's reaction	
Cell shape	Rods
Spore (s)	-
Motility	+

Growth temperatures	
4, 10, 25, 30, 37, 42, 55 ^{0}C	-, -, +, +, +, +, -
Growth pH's	
5, 6, 7, 8, 9, 10, 11, 12	-, +, +, +, +, +, +, -
Growth on NaCl (%)	
0.5, 2.0, 4.0, 6.0, 8.0, 10.0, 11.0, 12.0	+, +, +, +, -, -, -, -
Tests	
Catalase test	+
Oxidase test	+
Voges Proskauer test	-
Casein hydrolysis	-
Citrate	+
Nitrate	-
Arginine dihydrolase	-
Gelatin hydrolysis	-
Starch hydrolysis	-
Esculin hydrolysis	-
Tween 20	+
Tween 40	+
Tween 60	+
Tween 80	+
DNase	-

Acid Production from	
Cellobiose	-
Trehalose	-
Fructose	+
Maltose	+
Dulcitol	-
Sucrose	+
Dextrose	+
Raffinose	+

4.1.3.2 Pigment production and characteristics of the produced melanin

The isolated bacterium was incubated in marine broth and vegetable waste up to 48 h for melanin production. Here, vegetable waste from cabbage leftovers is selected as a growth medium as it satisfies the major micronutrient append as that of marine broth. Prior to the addition of inoculum, vegetable waste was supplemented with 1.9 % NaCl to maintain salinity as that of marine broth.

Pigment production was tested on three different media i.e., marine broth, blend (marine broth and vegetable waste - (10:90; 20:80 and 30:70 in v/v %), and diluted marine broth 30 % v/v. Fig. 4.1.3a shows the visible pigment production at significant amount was observed in 30:70 blend medium. This was selected for further study for economical pigment production Additionally marine broth – vegetable waste blend in 30:70 ratio also used as melanin production medium for comparison. As melanin pigment started to produce at proportion

(30:70) of blended medium; this was selected for further study as it is having maxmium proportion of vegetable waste and minimum marine broth.

The dark brown to black color (Fig. 4.1.3b) of the marine broth medium indicates more melanin production by the bacterial isolate than the marine broth - vegetable waste blended medium (Fig. 4.1.3c). The sole vegetable waste was not given any color but has cream slimy growth appearance after incubation of 2 days. The observed behaviors indicate melanin production was strictly medium dependent (i.e. marine broth here). The influence of medium was clearly evident from Fig. 4.1.3b and 4.1.3c where a 30:70 ratio of marine broth - vegetable waste blend gave less intense melanin when compared to marine broth alone. The melanin produced from pure marine broth and marine broth - vegetable waste blend was found to be 5.35 ± 0.4 and 2.79 ± 0.2 mg/mL after 72 h of incubation. The melanin from blended medium was found to be ~ 0.52 times lesser than pure marine broth.

However melanin from both sources after purification (by acid treatment) looked alike in appearance. The physical appearance of the purified melanin was also shown in Fig. 4.1.4 with a true black color typical of melanins in general [14]. The produced melanin was insoluble in water, ethanol, chloroform, acetone, benzene and slightly soluble in phenol and 1N NaOH. The melanin was precipitated with 6 N HCl and decolorized with the addition of H_2O_2. The observed features when compared with previous reports indicate the synthesized melanin was similar to that of bacterial melanin in properties [1].

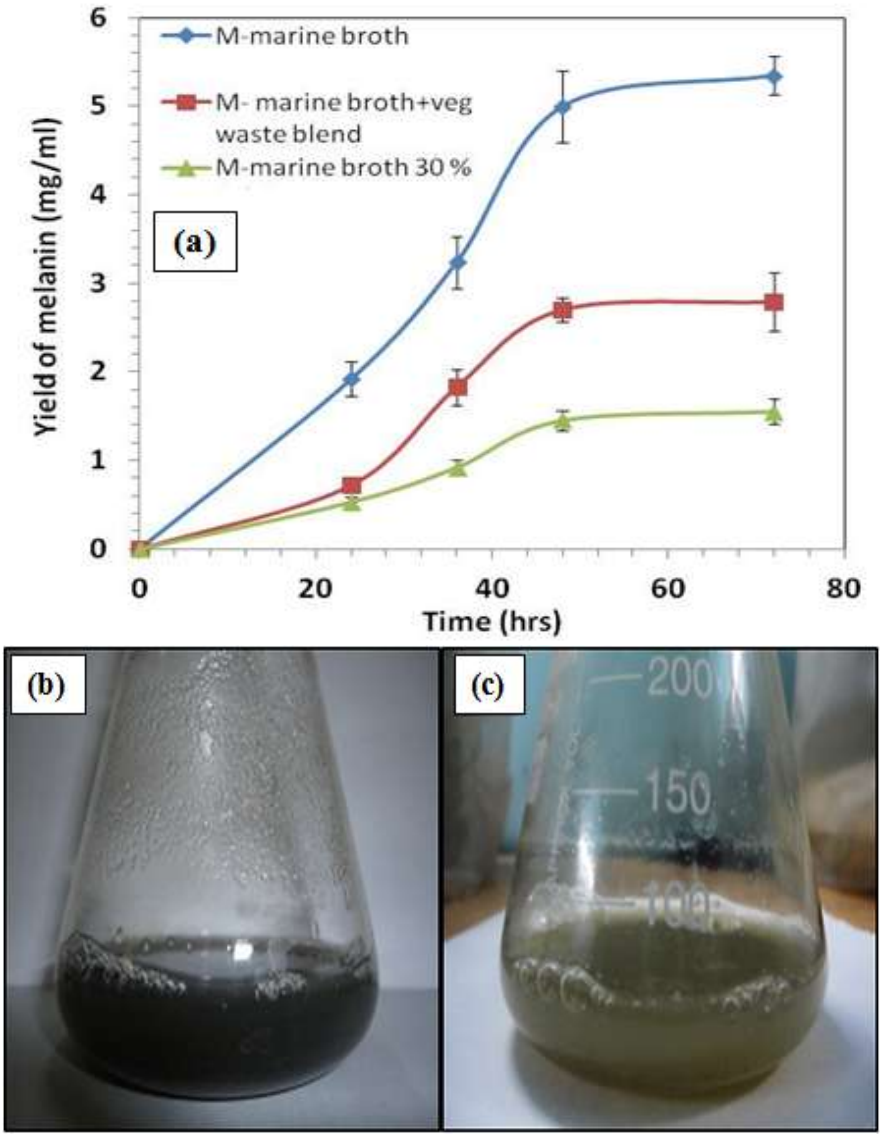


Fig. 4.1.3. Melanin production on various media: marine broth, blend (marine broth and vegetable waste - (10:90; 20:80 and 30:70 in v/v %), and diluted marine broth 30 % (a). M indicates melanin in (a). Dark brownish black colonies due to melanin production in sole marine broth (b) and in marine broth – vegetable waste blend medium (c).

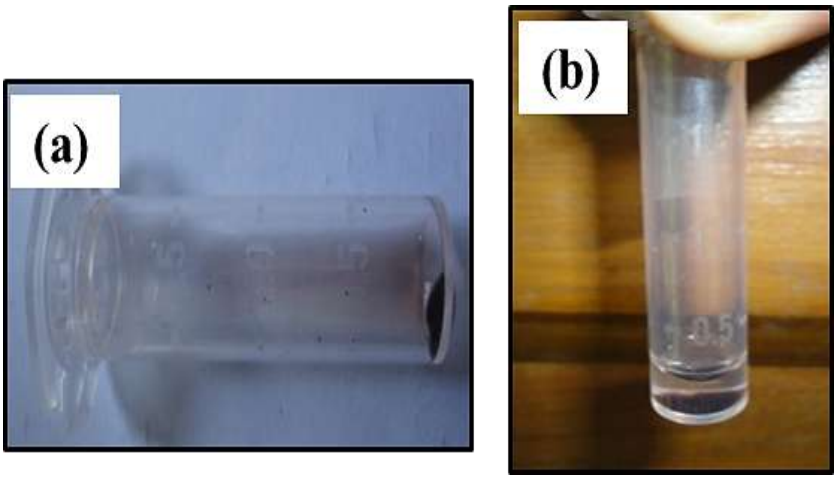


Fig. 4.1.4. Acid treated (a) and purified melanin (b) after centrifugation.

4.1.3.3 Spectroscopy, SEM/EDX and IR analysis of melanin

For a detail, inference and structural elucidation UV-visible spectroscopy, SEM/EDX and FTIR analysis were performed for the purified melanin pigments from two different media. The UV-visible wavelength scan showed the absorption was highest at the UV region of 200 to 300 nm, but diminished towards the visible region (Fig. 4.1.5) for both the melanins obtained. This phenomenon is characteristic to melanin and was due to actual complex structure of melanin [1].

Fig. 4.1.6 shows the SEM image of the purified melanin. The appearance from the figure suggests that the material was an amorphous deposit with no differentiable structures, similar to past reports of purified bacterial melanin [3]. Furthermore, reported studies show the ability of *Pseudomonas sp* (*P. aeruginosa* and *P. stutzeri*) to produce pyomelanin type melanin, where the pathway of synthesis includes catabolism of tyrosine via p - hydroxyphenylpyruvate and homogentisic acid (HGA) [2],[5]. To know the compositional examination of the obtained melanin, EDX analysis was performed and shown in Fig. 4.1.7.

The analyses revealed that the majority composition of the purified melanin from marine broth alone is of C, O with ~ 66 and 30 weight % and minor S content with ~3.85 %. While, melanin from blended medium showed C, O with ~ 35.62, 50.29 weight % and minor Ca content with ~14.09 %. Some peaks of Fig. 4.1.7 are undetectable as EDX may not be a reliable method to quantify elements in low weight % [3]. This result serve as an additional support which reflects the purity of the melanin produced. The compositional variation of melanin pigments might be due to the change in medium compositions.

FTIR spectroscopic analysis was performed on the acid treated purified melanin pigments to know the information about functional groups and structure. Fig. 4.1.8 shows the IR spectrum of melanins pressed into KBr disks. Similar spectral pattern from Fig. 4.1.8a and 4.1.8b indicate both melanin pigments obtained are having similar peaks corresponding to equivalent functional groups. The details of both spectra are as follows:

A broad absorption at 3373 cm^{-1} indicate the presence of – OH and NH_2 groups and small band at 2918 cm^{-1} can be assigned to stretching vibration of aliphatic C-H group [15]. The characteristic strong band at 1625 cm^{-1} (between 1650 - 1620 cm^{-1}) attributed to vibrations of aromatic ring C=C of amide I C=O and/or of COO- groups. Bands at ~1400 to 1500 cm^{-1} can be due to aliphatic C-H groups and weak bands below 700 cm^{-1} ascribed to alkene C-H substitution in the melanin pigment [1]. The observed IR patterns for the purified melanins were similar to the earlier reported DOPA-melanin study [4].

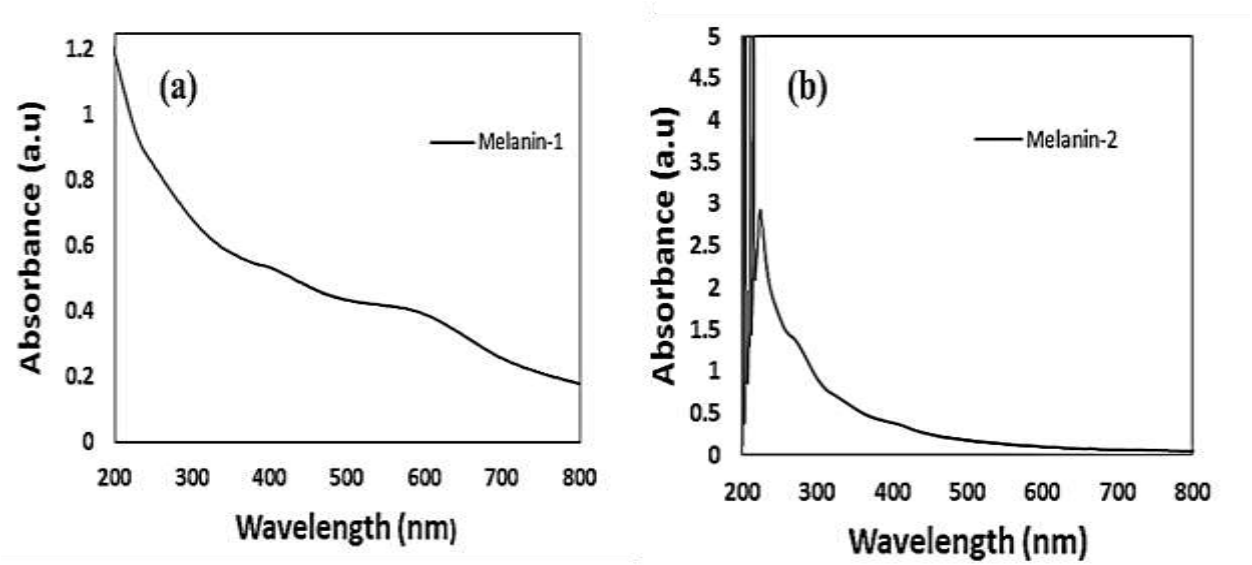


Fig. 4.1.5. UV-visible spectral properties of melanin pigment obtained from marine broth (a) and marine broth –vegetable waste medium (b).

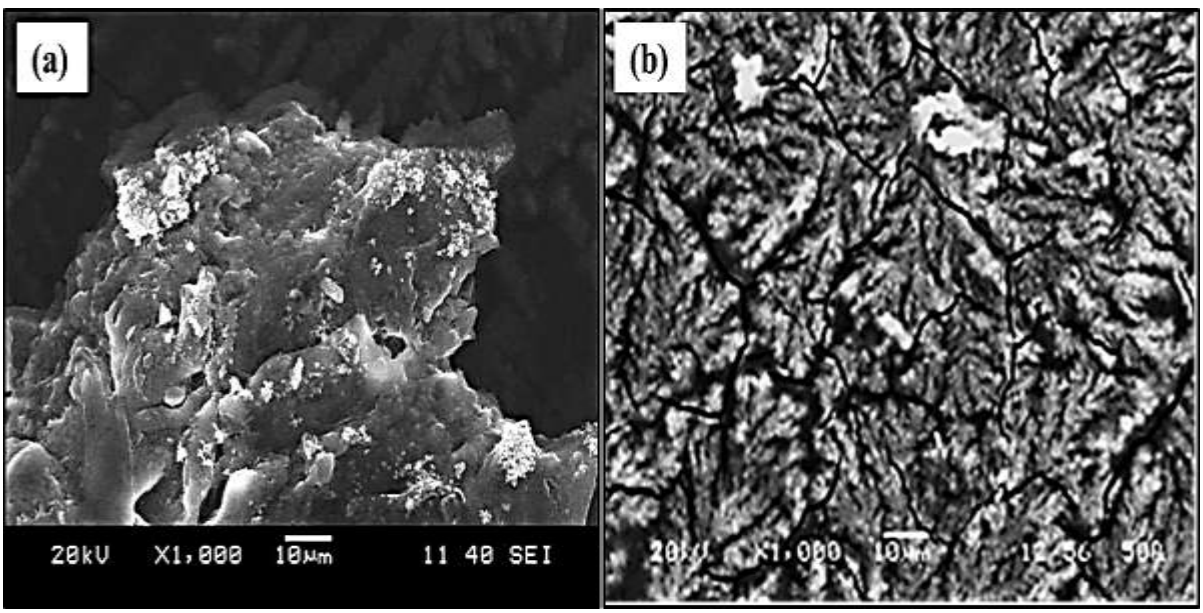


Fig. 4.1.6. SEM images of purified bacterial melanin from a) marine broth and b) marine broth – vegetable waste medium.

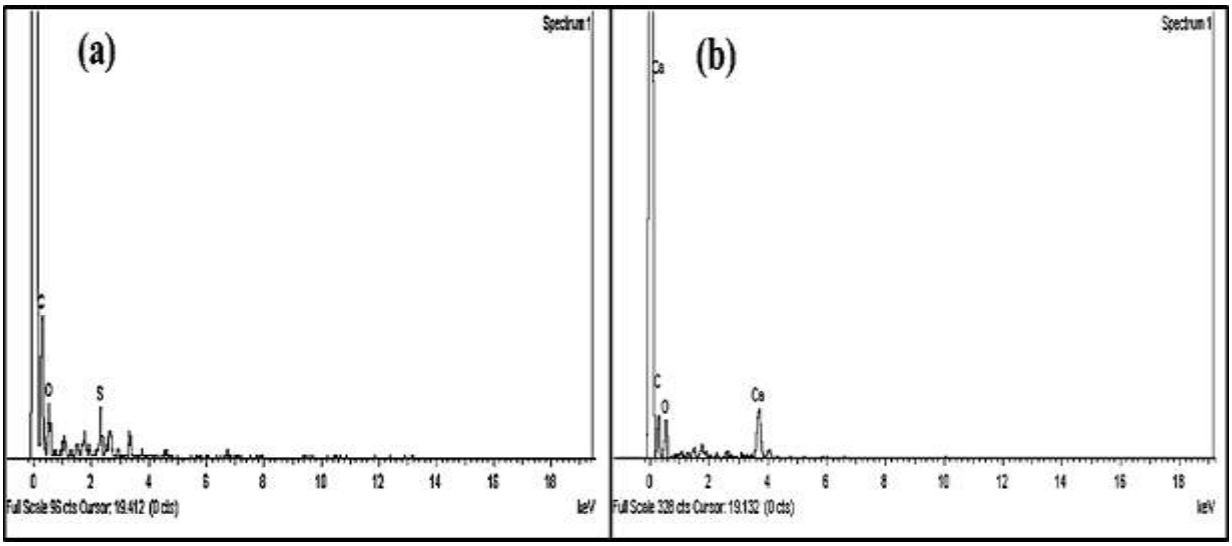


Fig. 4.1.7. EDX analysis of elemental composition of melanin from a) marine broth and b) marine broth – vegetable waste medium.

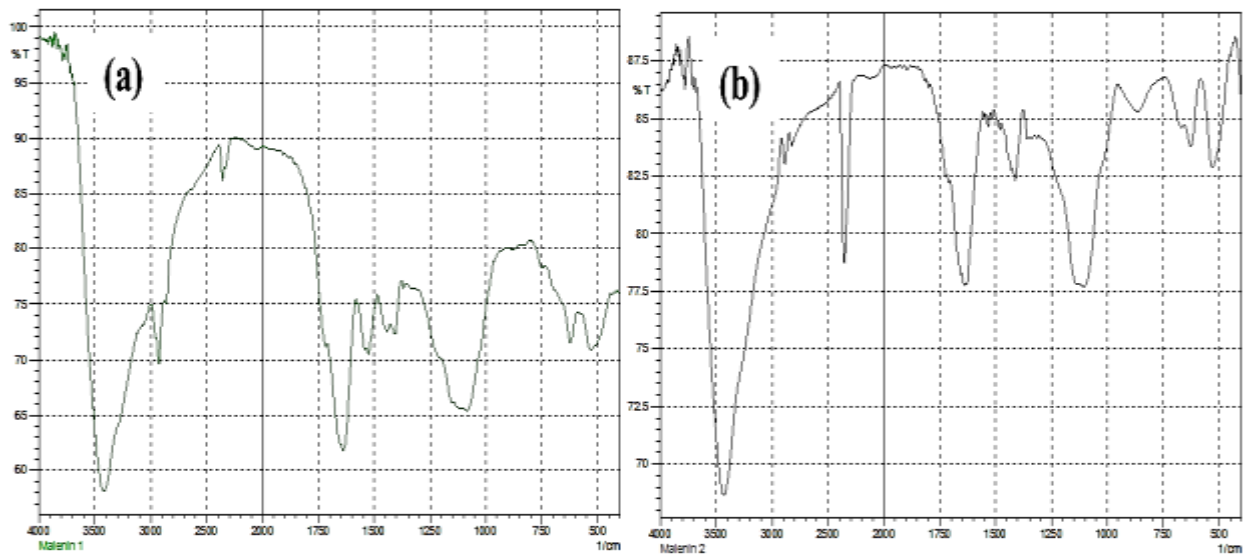


Fig. 4.1.8. FTIR spectrum of the melanin pigment obtained from a) marine broth and b) marine broth – vegetable waste medium.

4.1.3.4 DPPH assay

Melanin particle were found to possess antioxidant property in biological systems. It can scavenge free radicals and has the ability to sequester redox active metal ions [12]. Free radical scavenging activity was evaluated by performing *in-vitro* DPPH assay. Reduction of absorbance at 516 nm supplied with different melanin doses was shown in Fig. 4.1.9a. The colored DPPH solution faded and turned dull during the course of incubation of 3 days (Fig. 4.1.9 insert). This may be due to the reduction of the DPPH molecules and electron transfer from melanin suspension.

Fig. 4.1.9a also indicates a nonlinear pattern of DPPH reduction for various melanin doses used. A sharp change in absorbance up to 48 h for the used melanin concentrations indicates that the rate of reduction is rapid at initial stages. The diminished behavior (from Fig. 4.1.9a) beyond 48 h indicates the maximum threshold reduction by a particular melanin dose used. The reduction in spectral behavior at various time intervals (in days) was shown in Fig. 4.1.9b for a melanin dose of 44.7 µg/mL. Moreover from Fig. 4.1.9a, we can plot % scavenging activity with respect to melanin dosage after a residual period of 72 h (Fig. 4.1.9c). Henceforth, the minimum time period required by the melanin molecules for the maximum DPPH reduction and dose dependent scavenging activity were successfully valued in the present study.

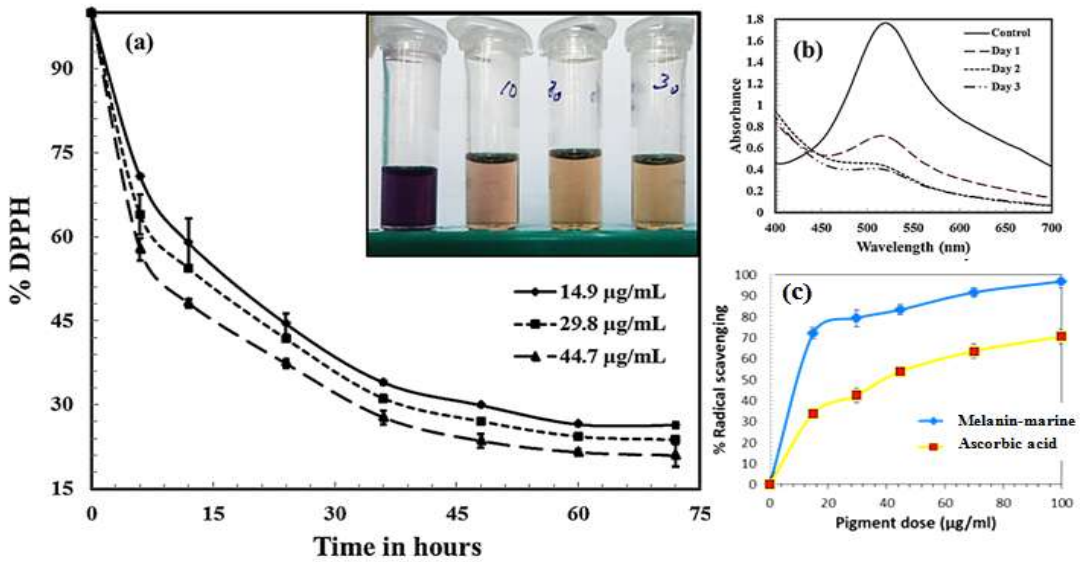


Fig. 4.1.9. DPPH radical scavenging activity of synthesized melanin pigment with various doses (a) and UV-vis absorption spectrum of melanin (44.7 µg/mL) - DPPH at different days along with control containing no melanin (b). Dose dependent scavenging activity of the synthesized melanin (c). Insert of (a) shows the melanin doses 0, 14.9, 29.8 and 44.7 µg/mL to 0.1 mM DPPH from left to right.

4.1.3.5 XRD analysis

The XRD spectrum of bacterial melanins and purchased melanin are shown in Fig. 4.1.10a. The spectra of melanins are characterized by a broad peak which is commonly seen in amorphous and disordered materials centered at about 24. The observed 2θvalues and crystallite sizes of the produced bacterial melanins are very close to synthetic (Fig. 4.1.10b). The inter layer spacing *d*, is calculated according to the Bragg equation.

$$2d \sin\theta = m\,\lambda.$$

where θ is diffraction angle, m is diffraction order and λ is X- ray wave length by considering first order diffraction (m = 1). The values of d are in good agreement with reported values of

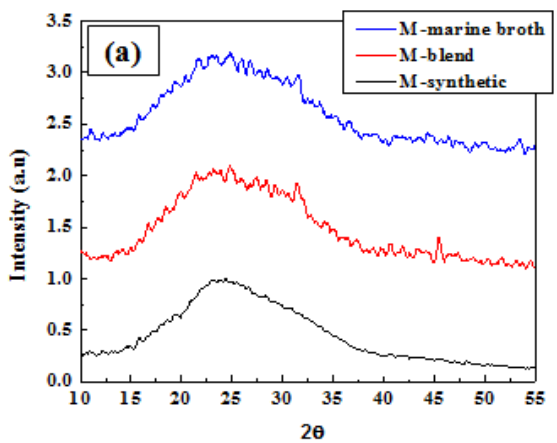
the inter layer spacing in the stacked sheets model of the melanin [16]. An estimate of average grain size of melanins was calculated from the Dedye - schrerrer equation [16].

$$D = 0.9\,\lambda/(FWHM.cos\theta)$$

where FWHM is full width at half maximum of diffraction peak. The closeness of the grain size values indicates the quality of the purified bacterial melanins. Furthermore % crystallinity was also calculated for the stated melanins by considering glass substrate as background. The calculation is as follows:

$$\%\,\text{crystallinity} = \frac{(\text{total area - background profile area})}{(\text{total area})} \times 100$$

% crystallinity values from Fig. 4.1.10c indicated that the bacterial melanin from blend and marine broth were less crystalline than the synthetic melanin studied. Lack of crystallinity is significant sign of consistent physical property of melanin [17]. And the above results state that the obtained bacterial melanins are of high pure and amorphous.



(b)

	2θ	d value	crystallite-D (A°)
M-synthetic	24.325	3.656	6.689
M-marine broth	24.75	3.594	5.711
M-blend	24.81	3.586	5.689

(c)

Sample	Peak area	% Crystallinity
M-blend	16.88	2.48
M-marine broth	17.64	6.71
M-synthetic	20.25	18.72
glass	16.46	

Fig. 4.1.10. (a) X-ray diffractograms of the produced melanin (M-marine broth, M-blend) and purchased melanin (M-synthetic). (b) Interlayer spacing (*d*-value) and crystallite sizes and (c) % crystallinity of the different melanins.

4.1.3.6 SPF values determination

The determination of SPF values for samples (bacterial and purchased melanin) was made through the UV spectrophotometric method and the Mansur equation was applied [13]. The results of different melanins were given in Table 4.1.2. As melanins are known for their photoprotective role [18], the comparable SPF values of the bacterial melanins to that of synthetic melanin (Table 4.1.3) indicate the produced melanin has significant photoprotection activity.

Table 4.1.2. Normalized product function used in the calculation of SPF by Mansur equation.

Wavelength (nm)	EE x I (normalized)	Abs(λ)		
		M-MB	M-MB+VW	M-control
290	0.015	1.776	1.844	1.980
295	0.082	1.656	1.694	1.852
300	0.287	1.536	1.570	1.733
305	0.328	1.411	1.461	1.623
310	0.186	1.297	1.374	1.522
315	0.084	1.207	1.306	1.430
320	0.018	1.149	1.260	1.345
M-melanin; MB-marine broth;VW-vegetable waste				

Table 4.1.3. SPF values of different melanins.

S.No.	sample	SPF value
1	Purchased melanin	59.336 ± 0.006
2	Melanin-marine broth	51.756 ± 0.008
3	Melanin-vegetable waste	53.738 ± 0.004

4.1.3.7 The metal binding capacity of melanin

The metal binding capacities of melanin from vegetable waste was determined by assessing its ability to compete with ferrozine for the ferrous ions. The concentration dependent metal chelating activity was shown in Fig. 4.1.11. The reduction in spectrum with increase in melanin dose (Fig. 4.1.11a) indicates that melanin compound was interfering with the formation of ferrous and ferrozine complex. The dose dependent activity was also clearly evident upon plotting % metal chelation effect against different melanin doses. Results show the better chelating effect of melanin than ferrozine towards ferrous ions and maximum effect (~ 55 % chelation) was observed in a dose of 0.146 mg/mL (Fig. 4.1.11b).

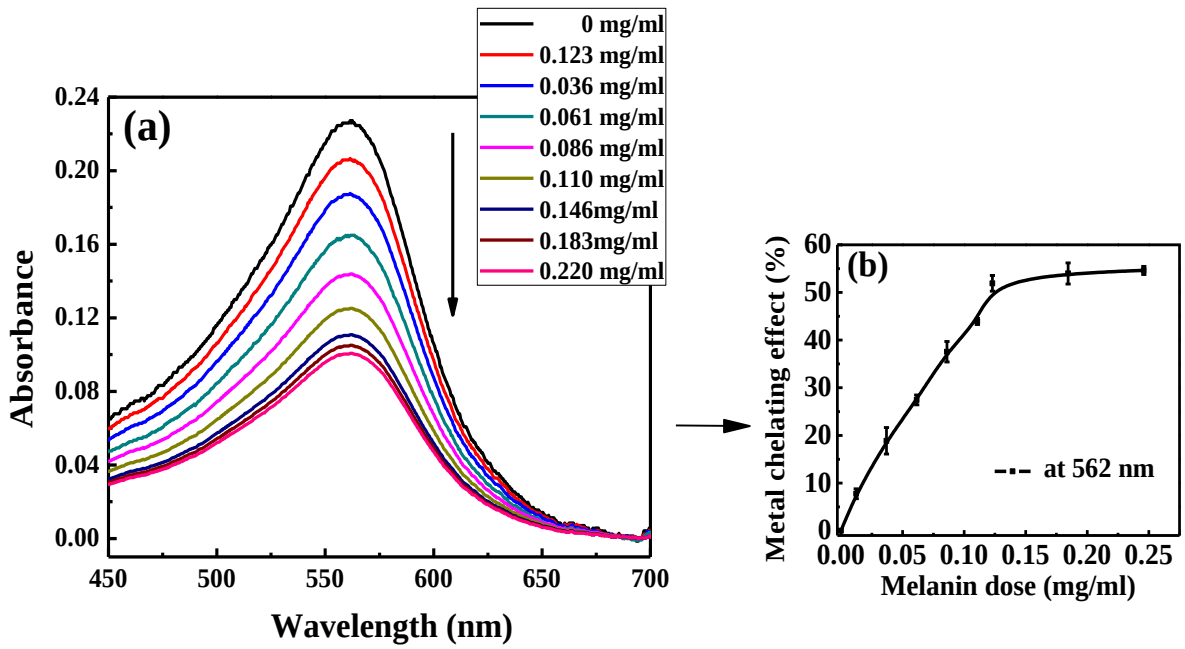


Fig. 4.1.11. Metal ion chelation effect of the melanin (produced from vegetable waste) (a) monitored via complete spectrum and (b). at a fixed absorption maximum

4.1.4 Conclusions

Though different marine isolates were used, it was found that the sample 1 contains the melanin producing bacterium and was found to be *Pseudomonas guinea*. Upon investigation for the melanin production in different media, 5.35 ± 0.4 and 2.79 ± 0.2 mg/mL pigment was produced when cultured in marine broth alone and blended medium (vegetable waste and marine broth), respectively. Pigments produced from the different nutrient sources have shown different elemental compositions with varying weight %. Moreover, the FTIR analysis revealed that functional groups were conserved in both the melanins and were appeared to be same. The compositional variation in the pigments might be due to the change in the media composition although *P.guinea*, only was used in both the cases. Furthermore, the produced melanin noticed to have efficient free radical scavenging activity (of a model DPPH radical) and also exhibited photoprotective and metal ion chelation activity. This study confirms that the antioxidant melanin pigment can be produced from the cheaper substrates without any functional variation.

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4.2 Melanin by Soil Microbial Isolate on Fruit Waste Extract: Optimization of Key Production Parameters

4.2.1 Introduction

Melanins are the natural pigments which have their presence in animals, plants and in most of the microorganisms [1]. They are the dark colored negatively charged high molecular weight pigments which are formed due to polymerized phenolic and/or indolic compounds. These complex polymers are amorphous in nature and shows solubility in neither aqueous nor organic solvents. They showed resistance to concentrated acids and are susceptible to bleaching by oxidizing agents [2]. They play a vital role in defense and protection mechanisms that improve the survival and competitiveness of the organisms [3]. Melanin is known for its absorption capacity of radiation of all wavelengths with an optimum absorbance at UV range [4] which prevents photo induced damage. Hence it is used in the preparation of photo absorbing optical lenses and in bioplastics. Besides photo protection it has versatile biological activities such as radical scavenging, antioxidant, antitumor, anti-inflammatory [5] and as immune stimulating agent [6].

Melanin obtained from microbes has great advantages over melanin from animals and plants. Microorganisms don't cause the problems of seasonal variations and are selected arsenals as they modify them according to the medium and conditions provided to them [7]. Targeting melanogenisis in microbes may help to discover antimicrobial drugs. For example, melanins produced by *Cryptococcus neoformans* and *Burkholderia cepacia* offer virulence and contribute to the growing resistance of these pathogenic bacteria towards antibiotics [2, 8]. The melanin synthesized by microbes shows metal chelating ability too (sorb the radioactive wastes uranium) [9]. There are reports that showed the anti HIV properties of melanin and their role in photo voltage generation and fluorescence studies [10-11]. Therefore all these properties of melanin make them unique and are widely used in cosmetic, sunscreen protection creams, eye glasses, pharmaceuticals, and food industries

Eying on the potential uses and increasing demand for the melanin pigment there is a need to conduct studies on the production of melanin from microbes. There are reports on melanin production from various microorganisms including *Bacillus* species which are well known for their pigment production ability in various stress environments [4, 12]. Selection of substrate for melanin production has economic importance. For instance till date expensive substrates like NCM media [4], LB (Luria-Bertani) media [12], minimal media supplemented with L-tyrosine [13], amino acids enriched tryptone broth agar [14] and so on [15-16] were used for high yield of melanin. Owing to the economy and practicability of the melanin production process; the need to use economically feasible substrates along with optimization of key parameters is needed. Taguchi method [17] is a systematic technique of design and analysis of experiments that has been employed successfully in recent years to design and

improve product quality economically [18], CCD design approach has been used to fit a polynomial model.

In this study, a bacterium capable of producing melanin was isolated from garden soil and subsequently characterized. The strain was cultivated in the fruit waste extract (FWE) [as sole source of energy] to produce significant amounts of melanin. The key parameters in melanin production were identified and optimized using simple two step Taguchi and CCD (central composite design) approach. Upon purification and characterization, the obtained melanin was tested for *In vitro* sun protection effect, free radical scavenging and metal chelating activities.

4.2.2 Materials and methods

4.2.2.1 Chemicals and microorganism

DPPH (2,2-diphenyl-1-picrylhydrazyl), purchased from HiMedia chemicals, Mumbai, India. Ascorbic acid was purchased from Merck, India. Ferrozine and melanin (synthetic) was purchased from Sigma-Aldrich, India. Ethanol, NaCl, NaOH, HCL are from Merck, India and all other chemicals used were of analytical reagent grade throughout the study. Ultrapure water was used for the experiments and aseptic conditions were maintained wherever necessary.

The microorganism used in this study was isolated from garden soil in front of Department of Chemical Engineering, National Institute of Technology, Rourkela, India using serial dilution technique on a nutrient agar (NA) [19]. Using 10^7 dilution soil samples on NA plates. Melanin producing organism was identified. It was separated by observing a

diffusible black pigment on NA plates after 24 h. The isolated culture was preserved on NA slants at 4 ^{0}C and sub-cultured at monthly intervals.

4.2.2.2 Substrate preparation

Fruit waste was obtained from a fruit juice shop of a local market. The material used is from single batch i.e. used in all the experiments to minimize the disturbances in the results due to variations in composition. The waste contains major portion of pine apple and orange waste and minor portions of pomegranate waste. Fruit waste includes extracted carpels of oranges, core of pineapples, and crushed seeds along with arils of pomegranate. The soluble sugars were extracted from 1 kilogram of fruit waste by adding 2 liters of distilled water and boiled at 100 ^{0}C for 30 minutes. The resultant straw color fruit waste extract (FWE) was filtered and stored at 4 ^{0}C for further experimentation. The prepared FWE with a flow sheet of preparation was given in Fig. 4.2.1.

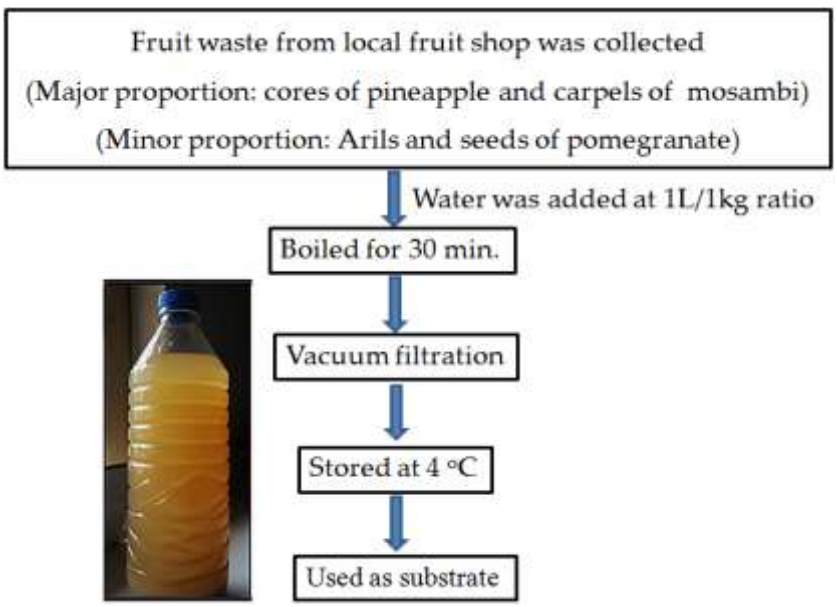


Fig. 4.2.1. Steps involved in substrate preparation along with the prepared FWE as figure insert.

4.2.2.3 Production and purification of melanin

Nutrient broth (peptone-5g/L, beef extract-3g/L, NaCl-5g/L) was used for inoculum preparation and FWE was used as production medium for melanin. About 10 µL (10^8 CFU/mL) culture suspension was added to FWE medium in 250 mL flasks with a working volume 50 ml. The medium was then incubated at 30 ^{0}C on a rotary shaker moving at 200 rpm for 24 h. A dark pigmented and nearly opaque FWE medium was observed (Fig. 4.2.2a). After the incubation time, the medium was centrifuged using REMI-RM12C, India centrifuge at 8000 rpm for 15 min to separate the broth (supernatant) and the cells. The solid pellet of cells were separated and suspended in distilled water. The cells were further centrifuged to collect the supernatant. Melanin was extracted from the overall supernatant by acidification with 3 N HCl to pH-2 and allowed to stand for 48 h initially at room temperature. This process was repeated for 7 more days until no precipitate was obtained. The obtained suspension was boiled for 5 min to prevent the formation of melanoidins. As a final point, crude pigment pellet was collected after centrifugation at 4000 rpm for 15 min.

4.2.2.4 Parameters optimization using Taguchi and CCD design

Preliminary idea on growth conditions, suggests Taguchi method to be employed for the optimization of culture conditions for high yield melanin pigment production. Optimization of three vital factors like pH, temperature and agitation in 6-3-3 levels respectively was done as a starting point of the study (Table 4.2.1). Then Taguchi method was performed by 18 different experiments by using L18 orthogonal array as shown in Table 4.2.2. Shown values of the obtained melanin (mg/mL) are the average of results of two replicates. Based on the obtained results, the optimum conditions of the used parameters were identified and an analysis of variance (ANOVA) for the obtained results was investigated.

Once the critical factors were identified, in addition to the above, a central composite design (CCD) for independent variables was used for further optimization. Two variables at two levels were used to fit a polynomial model. A two level full factorial is performed with a model equation designed such that the variance of Y is constant for all points equidistant from the center of the design. Minitab (14.0) statistical software package was used in the experimental design and data analysis. Response surface graphs were obtained to know the effect of the variables, individually and in combination, and to determine their optimum levels for maximum melanin production. All trials were performed in duplicate, and the average melanin yield was used as response Y.

4.2.2.5 SPF/DPPH/metal chelating assay

In vitro sun protection factor

As per the adapted method, spectra of melanin samples were collected over the spectral range 400-280 nm with 1 nm data point resolution on a UV-visible UV-3200 double beam spectrophotometer (LABINDIA analytical Instruments Pvt Ltd, India). The SPF values of melanin from microbial isolate and purchased melanin were determined using Mansur mathematical equation (1).

$$\mathrm{SPF} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}(\lambda) \times \mathrm{I}(\lambda) \times \mathrm{Abs}(\lambda) \qquad (1)$$

Where, SPF=Sunscreen protection factor; EE(λ)=Erythremal effect spectrum, I(λ)=Solar intensity spectrum; Abs(λ)=Absorbance of sunscreen product; CF=Correction factor (=36.2 for SNOW LOTUS® SPF 15 and 30) Huang et al.[20]

DPPH assay

The radical scavenging activity by melanin pigment was investigated by modified method of Ju et al. [21]. Primarily 1 mL of (15-100 μg/mL) microbial melanin/standard (Ascorbic acid) was added to 2 mL of DPPH in ethanol. After keeping for 30 minutes at 37^{o}C the absorbance at 516 nm was measured using UV-spectrophotometer with reference blank samples. The experiment was performed in duplicate. The absorbance of DPPH as control was measured at 516 nm. Lower absorbance of the reaction mixture indicated higher radical scavenging activity. The scavenging effect was measured using equation (2).

DPPH inhibition (%) = [(Control absorbance -test absorbance)/Control absorbance] X 100 (2)

Determination of metal chelating activity

The chelation of ferrous ions by the melanin pigment was estimated by the method of Huang et al.[20]. Different concentrations of melanin were mixed with a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All the tests and analysis were performed in duplicate and averaged. The inhibition of the melanin pigment metal chelating activity in percentage (%) was calculated using equation (3).

Metal chelating effect (%) = $[(A_0-A_1)/A_0]$ X 100 % (3)

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of the sample of the melanin pigment or standards. The control contains $FeCl_2$ and ferrozine.

Table 4.2.1. Factors and their levels which were studied by Taguchi approach.

Factor	*Levels*					
	1	2	3	4	5	6
pH	4.3	5	6	7	8	9
Temperature (^{o}C)	15	30	45			
Agitation (rpm)	90	140	180			

Table 4.2.2. Levels of three different factors, applied in each of 18 trials with the obtained results.

pH	Temperature	Agitation	Melanin (mg/mL)
4.3	15	90	0.001
4.3	30	140	0.01
4.3	45	180	0.011
5	15	90	0.08
5	30	140	0.124
5	45	180	0.11
6	15	140	0.309
6	30	180	0.331
6	45	90	0.328
7	15	180	0.522
7	30	90	0.655
7	45	140	0.577
8	15	140	0.356
8	30	180	0.446
8	45	90	0.428
9	15	180	0.219
9	30	90	0.231
9	45	140	0.218

4.2.2.6 Analytical methods

XRD analysis was performed using the CuKα radiation (λ=1.5406 A^o) X-ray diffractometer [Philips (PW1830 HT)], in the range of 20–90^0 (2θ) at 0.05°/s with an accelerating voltage 35 kV and applied current (30 mA). The absorption spectra of the purified melanin solutions at room temperature were obtained by UV-visible spectrophotometer. Structural functional groups were identified by FTIR, [Bruker**,** Germany] equipped with attenuated total reflectance (ATR) mode with zinc selenide (ZnSe) crystal.

4.2.3 Results and discussion

4.2.3.1 Strain selection and pigment production

The melanin producing soil microbial isolate from NA plates was carefully separated and cultivated on fresh agar plates (Fig. 4.2.2b) for 24 h. These colonies were examined microscopically for their morphology as shown in Fig. 4.2.2c. The isolated strain upon 16S rDNA sequencing identified a novel bacterial species *Bacillus safensis* strain ZJHD1-43 (GenBank Accession Number:**KF585035.1**). The phylogenic tree was constructed showing the position of isolate with reference to related strains in Fig. 4.2.2d. At usual conditions, FWE appeared to be most suitable medium for melanin production. An intense coloration of the medium from straw colour to brownish black was observed within 24 h at a pH of 7and with agitation of 100 rpm.

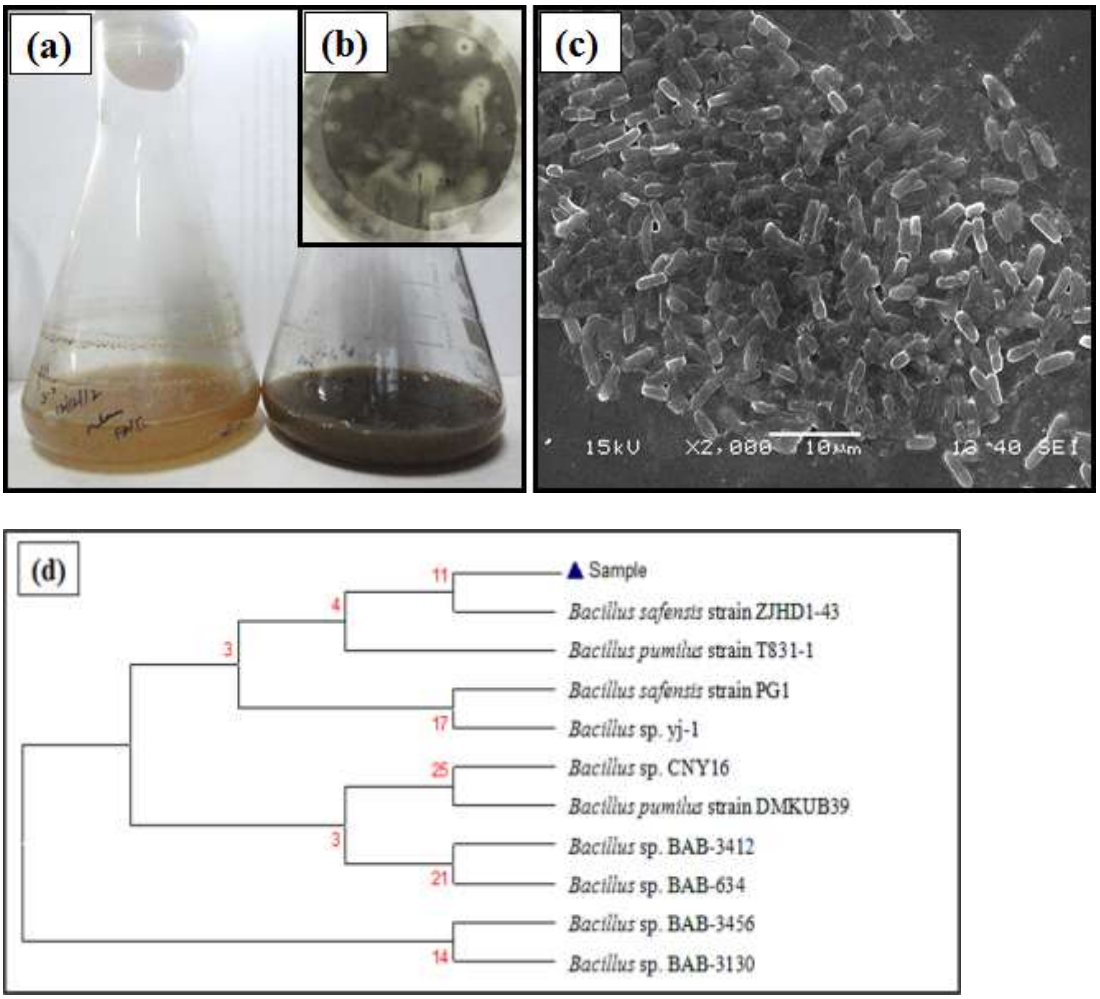


Fig. 4.2.2. (a) FWE before (left) and after melanin production (right) by the garden soil microbial isolate, (b) colonies with diffusible melanin on NA plates, and (c) SEM image of the microorganism. (d) Phylogenetic tree showing the position of the isolate ZJHD1-43 with reference to related strains.

4.2.3.2 Taguchi design for screening critical factors

Effect of pH, temperature and agitation were studied employing Taguchi method, which is a fractional factorial experimental design tool. Experiments performed at experimental conditions (pH 7; temperature 30 $^{\circ}$C; agitation 90 rpm) produced maximum melanin of 0.655

mg/mL on an average as shown in Table 4.2.2. Each of these factors such as pH, temperature and agitation influenced significantly on melanin production represented as "main effect" and illustrated in Fig. 4.2.3. Using ANOVA software tool, significance of two important factors pH and temperature was reflected as per Table 4.2.4. F value represents the relative contribution of estimated variance to residual variance. Large F value is desirable as small value indicates significance of pH in this optimization method. Again further confirmation of the significant effect is understood from P value. Using P-value prob > F test that indicates probability of F value that will be observed when $P < 0.05$. Thus we found that pH and temperature has significant influence in optimization of process conditions towards melanin production, whereas agitation has negligible effect.

Table 4.2.3. Analysis of variance of main effects of factors

Analysis of Variance for Means

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	5	0.659	0.659	0.131873	195.66	0
Temperature	2	0.008	0.008	0.004054	6.02	0.025
Agitation	2	0.001	0.0014	0.000714	1.06	0.39
Residual Error	8	0.0053	0.0053	0.000674		
Total	17	0.6742				

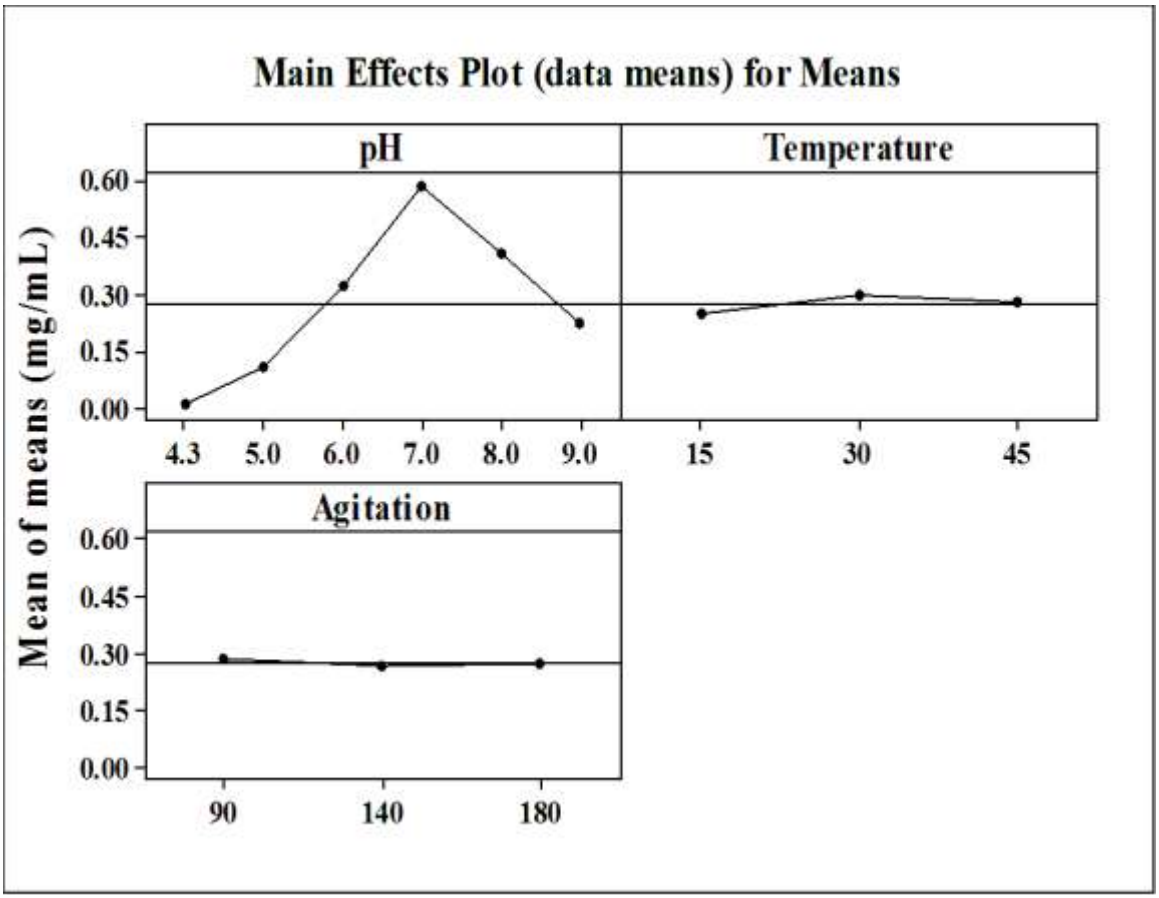


Fig. 4.2.3. Main effects of factors or average of obtained results (mg/mL) in which each factor is at a given level. For description of 'levels' refer to Table 4.2.1.

Furthermore Table 4.2.4 shows the suggested condition as predicted from the optimization tool. Statistical calculations predicted that at these conditions (Table 4.2.4) the melanin yield should reach 0.620 mg/mL. However this value is slightly less than and almost equals the value by trail no: 7 (from the array of experiments given in Table 4.2.2). Hence further investigation at the suggested conditions (Table 4.2.4) was discontinued.

Table 4.2.4. Optimum conditions suggested by statistical calculations after performing the tests

Factor	Level description	Level	Contribution (mg/mL)
pH	7	4	0.584–0.275=0.309
Temperature (^oC)	30	2	0.299 –0.275=0.024
Agitation (rpm)	90	1	0.287 –0.275=0.012

4.2.3.3 Optimization by response surface methodology (CCD design)

Optimization of process parameters was carried out using the CCD design with the parameters found to be significant from the Taguchi approach, including pH (X_1) and temperature (X_2). Table 4.2.5 represents the design matrix and the results of the 13 experiments carried out using the CCD design. The data obtained provided the regression model using ANOVA software.

$$Y = 6.7014 - 0.3367(X_1) + 0.2083\ (X_2) - 0.6048\ (X_1*X_1) - 0.8598\ (X_2*X_2) - 0.1175\ (X_1*X_2) \quad \text{---------(4)}$$

Where X_1 and X_2 represents pH and temperature respectively. The estimated regression coefficients from response surface analysis of the quadratic regression model (Table 4.2.6) demonstrate that equation. 4 is a highly significant model with goodness of fit R^2 - 0.982 and adjusted R^2 - 0.969. These values indicate that the model equation was adequate for predicting the melanin production under any combination of values of the variables.

Table 4.2.5. Experimental design matrix for the central composite design.

pH	Temperature	pH values	Temperature values	Melanin (mg/ml)
0	1	7	35	6.12
0	0	7	30	6.63
0	-1	7	25	5.54
0	0	7	30	6.78
0	0	7	30	6.83
1	0	7.5	30	5.62
-1	1	6.5	35	5.8
-1	-1	6.5	25	5.23
0	0	7	30	6.68
0	0	7	30	6.61
-1	0	6.5	30	6.55
1	1	7.5	35	5.02
1	-1	7.5	25	4.92

4.2.3.4 Interaction effects of variables and validation of the model

The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to identify the optimum conditions. The interaction effects and optimal levels of the variables were determined by plotting the three dimensional (3D) response surface curves. The response surface curve in Fig. 4.2.4a,b represents the interaction between

pH and temperature, which showed that the maximum melanin yield was obtained toward neutral pH while melanin yield was significantly affected with alkaline pH.

Validation was carried out under conditions predicted by the model. The optimum conditions predicted by the model are pH - 6.84, Temp - 30.7 ºC with yield of ~ 6.8 mg/mL and the actual yield obtained was 6.96 ± 0.6 mg/mL. The close correlation between the experimental and predicted values signifies the reliability of the response methodology (CCD design) over traditional optimization approach. In addition, the increased melanin production was observed with the optimized conditions than the initially used conditions. Additional to the above, yield of melanin under optimum conditions was observed at different time intervals and are shown in Fig. 4.2.4c.

Table 4.2.6. Estimated regression coefficients from the model equation.

Response Surface Regression: melanin (mg/ml) versus pH, Temperature

Term	Coef	SE Coef	T	P
Constant	6.7014	0.05127	130.714	0
pH	-0.3367	0.05041	-6.679	0
Temperature	0.2083	0.05041	4.133	0.004
pH*pH	-0.6048	0.07429	-8.141	0
Temperature*Temperature	-0.8598	0.07429	-11.573	0
pH*Temperature	-0.1175	0.06173	-1.903	0.099

S = 0.1235; R-Sq = 98.20%; R-Sq(adj) = 96.90%

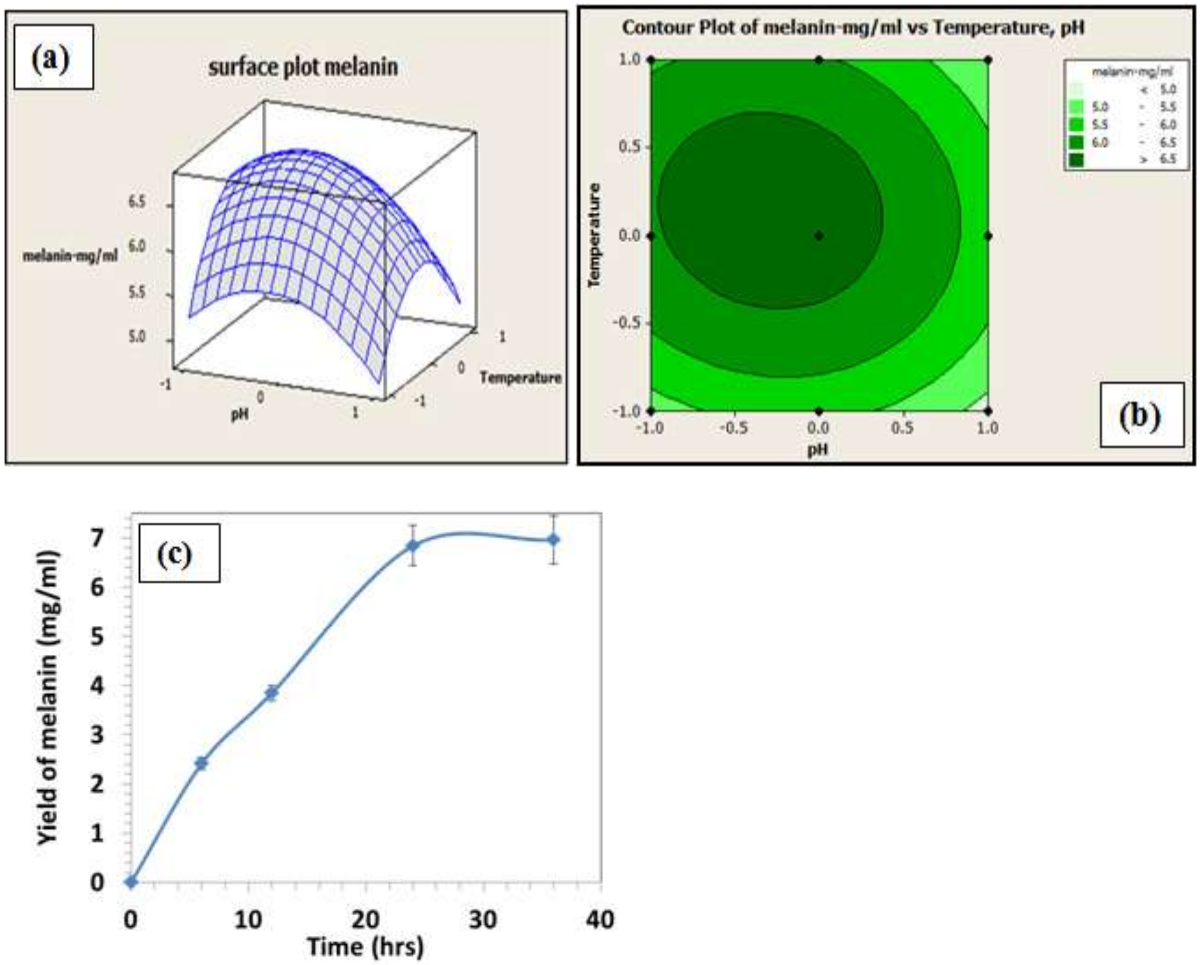


Fig. 4.2.4. Three dimensional response surface curves with surface plot (a) and contour plot (b) showing the effect of interactions of pH and temperature on melanin yield. (c) melanin yield at optimum conditions with respect to time.

4.2.3.5 Spectroscopy, IR and XRD analysis of melanin

UV- Vis analysis

The absorption spectrum of natural melanin is shown in Fig. 4.2.5a. The UV-visible wavelength scan showed that absorption was highest in the UV region (200 to 300 nm), but

diminished towards the visible region. This phenomenon is characteristic to melanin and was due to actual complex structure of melanin [1, 13].

FTIR analysis

IR spectroscopy is important for the interpretation of the structure binding capacity, affinity and sites of metal ions in melanin. Fig. 4.2.5b,c shows strong absorptions at 3500 cm^{-1}, 1700 cm^{-1}, 1300 cm^{-1} for standard melanin and for bacterial melanin obtained. The signals from 3600-2800 cm^{-1} attribute to the stretching vibrations of (O-H and N-H) the carboxylic, phenolic and aromatic amino functional groups of indolic and pyrrolic systems [22-23]. The spectral area between 1750 - 1550 cm^{-1}represents the bending vibrations of C=O. the OH bending of phenolic and carboxylic groups are present in 1400 - 1300 cm^{-1} [22].

XRD analysis

The XRD spectrum of bacterial melanin and purchased melanin are shown in Fig. 4.2.5c. The spectra of melanin are characterized by a broad peak which is commonly seen in amorphous and disordered materials centered at about 24°. The observed 2θvalues are 24.83º and 24.32º for bacterial and purchased melanin respectively (Fig. 4.2.5c). This peak is due to X- ray diffraction from parallel planer layers. The inter layer spacing *d*, is calculated according to the Bragg equation.

$$2d \sin\theta = m\,\lambda. \qquad (5)$$

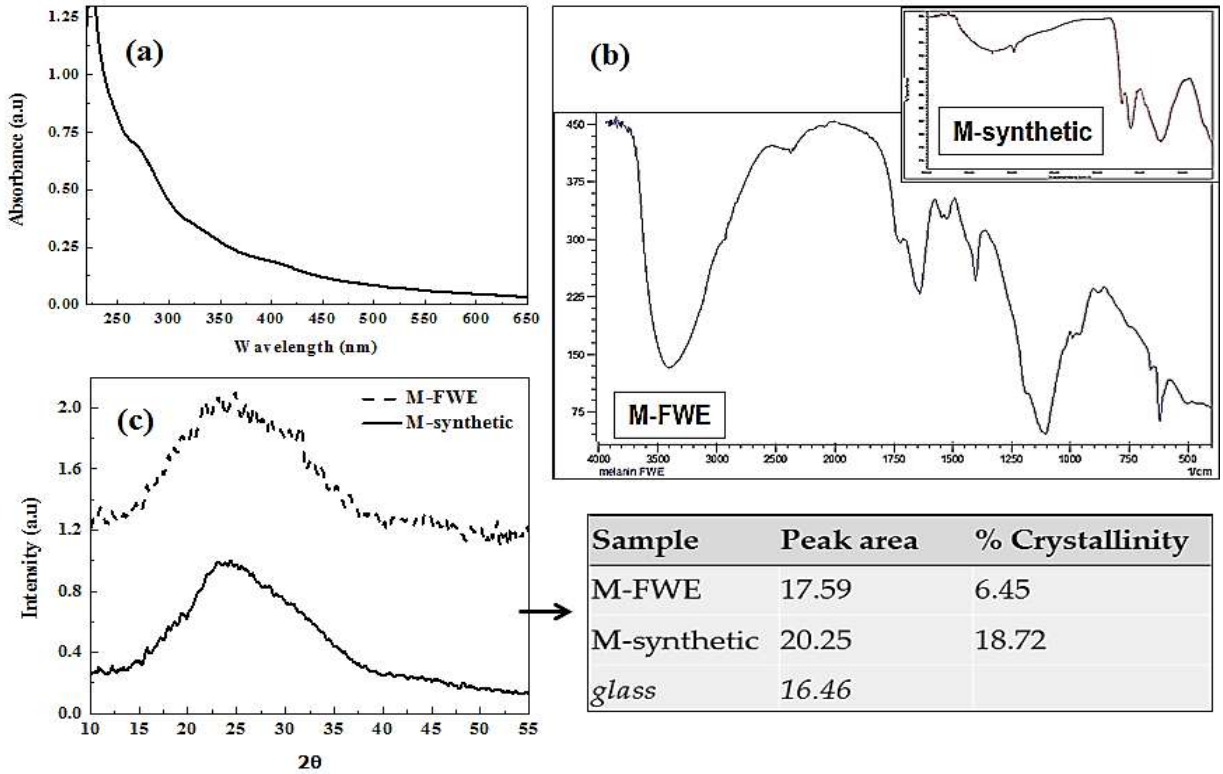
where θ is diffraction angle, m is diffraction order and λ is X- ray wave length by considering first order diffraction (m = 1) we obtained d values of 3.582 and 3.656 Aº for bacterial and purchased melanins respectively. The value of d is in good agreement with reported value of the inter layer spacing in the stacked sheets model of the melanin [1]. An estimate of average grain size of melanins can be calculated from the Dedye - schrerrer equation [1].

$$D = 0.9\,\lambda/(FWHM.cos\theta) \quad (6)$$

where FWHM is full width at half maximum of diffraction peak. The obtained D values are 0.668 and 0.568 nm for the bacterial and purchased melanins. The closeness of the grain size values indicates the quality of the purified bacterial melanin. Furthermore % crystallinity was also calculated for the stated melanins by considering glass substrate as background. The calculation is as follows:

$$\%\,\text{crystallinity} = \frac{(\text{total area - background profile area})}{(\text{total area})} \times 100 \quad (7)$$

Although both melanin samples exhibited the lack of structure in the diffraction pattern corresponding to any significant crystallinity, the % crystallinity values (Fig. 4.2.5c, picture indicated with arrow) further indicate bacterial melanin from FWE was far less crystalline when compared to the purchased melanin. Lack of crystallinity is significant sign of consistent physical property of melanin [24].



Sample	Peak area	% Crystallinity
M-FWE	17.59	6.45
M-synthetic	20.25	18.72
glass	*16.46*	

Fig. 4.2.5. (a) UV-visible spectrum of melanin pigment obtained from FWE. (b) FTIR spectra of standard melanin (upper) and bacterial melanin (lower). (c) X-ray diffractograms of the obtained melain (upper) and purchased melanin (lower) and % crystallinity of both are also shown as indicated by arrow.

4.2.3.6 SPF values determination

The determination of SPF values for samples (bacterial and purchased melanin) was made through the UV spectrophotometer using the Mansur equation [20]. The SPF value for melanin in FWE was 53.36 ± 0.009, while it was 59.34 ± 0.006 for purchased melanin.

As melanins are known for their photoprotective role [25], the obtained SPF values state that melanin from FWE might have profound protection effect against dermal damage related to photoaging as that of purchased melanin.

4.2.3.7 DPPH assay

DPPH accepts an electron to become a stable diamagnetic molecule. The ethanolic solution of DPPH (violet color) has got a strong UV absorbance at 516 nm. The presence of a reducing agent in this ethanolic solution pairs the odd electrons of DPPH radical and further the solution losses color stiochometrically and also the absorbance of the solution decreases at 516 nm. Reduction of absorbance at 516 nm and color of DPPH associated with different melanin doses was verified. The % increase in radical scavenging activity from Fig. 4.2.6a indicates the diminished behavior of the radical. The data obtained from Fig. 4.2.6a states that scavenging activity of the melanin was higher than the control ascorbic acid at each and every dose studied. This behavior shows 30 % enhanced reductive capability of the obtained bacterial melanin than ascorbic acid for a constant dose of melanin dose of ~100 µg/mL.

4.2.3.8 The metal binding capacity of melanin

The metal binding capacities of melanin from FWE was determined by assessing its ability to compete with ferrozine for the ferrous ions. The concentration dependent metal chelating activity was shown in Fig. 4.2.6b and its insert. The reduction in spectrum with increase in melanin dose indicates that melanin compound was interfering with the formation of ferrous and ferrozine complex. This suggests the better chelating effect of melanin and good ability to capture ferrous ions than ferrozine. Maximum effect (~ 64 % chelation) was observed for a dose of 0.2 mg/mL (Fig. 4.2.6c). The results suggest that the action of melanins as oxidation protection factors may be predominantly due to their iron binding capacity.

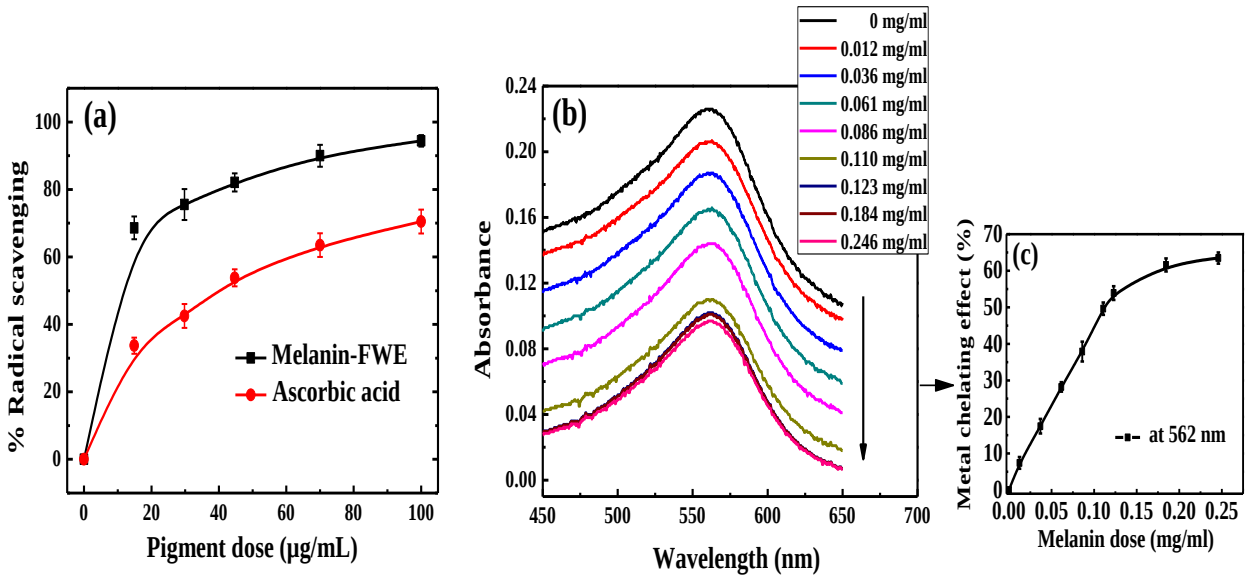


Fig. 4.2.6. (a) Dose dependent scavenging activity of the synthesized melanin from FWE and ascorbic acid as a control. (b) Metal ion chelation effect of the produced melanin in different doses (monitored spectrally) and (c) At a fixed absorption maximum.

4.2.3 Conclusions

From the results of this study, it is concluded that the use of two step statistical approach not only helped in locating the optimum levels of the most significant factors considered with minimum resources and time but also proved to be an useful and satisfactory method in melanin production-optimizing exercise. Thus, the optimization of vital nutritional parameters using response surface methodology significantly enhanced the yield of melanin on fruit waste extract has proved its feasibility for large-scale production by a garden soil isolate (*Bacillus safensis*). The melanin obtained in this study has photoprotective, radical scavenging and metal binding capacity which is of economic importance. So the *B. safensis* and fruit waste extract can be potential sources for melanin production.

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4.3 Carotenoid by *Bacillus clausi* Using Rice Powder as the Sole Substrate: Pigment Analyses and Optimization of Key Production Parameters

4.3.1 Introduction

Pigments are particulate solids that disperse into a medium without significant interaction in solutions [1]. Their wide applications in fields of food, cosmetics, paints, pharmaceuticals, textiles, glass etc. has contributed to its increasing demand [2]. Currently there are two methods employed so far to produce β-carotene; they are based on Wittig reaction developed by Badische Anilin and Soda Fabrik (BASF) and the second is based on Grinard reaction elaborated by Hoffman-La Roche [3-4]. However the microbial method has gained maximum interest as they are naturally produced and are environmentally friendly. Carotenoids are the secondary metabolites which are usually accumulate in the organisms during biotic and abiotic stress. Among all natural pigments, carotenoids represent the largest and most diverse class compounds known to mankind [5].

Carotenoids of microbial origin have found maximum application in the food and cosmetic industries which has exhibited increase in demand in recent years. Considerable research has been focused on these pigments as nutritional supplements with some $ 935 million to billion dollars market in 2005 [6-7]. Besides, carotenoids play a significant role in human health as precursors of vitamin A, scavengers of active oxygen, enhancers of *in vitro* antibody production, anticancer agents and so on [6, 8].

In view of high cost of current technology there is a need to develop low cost processes for the production of pigments which could replace the synthetic pigments in industrial scale. Production of pigments essentially depends on the type of substrate on which the microbe grows [9]. A variety of substrates are tested for carotenoids production using different microbes with special emphasis on cheaper substrates like maple sap [10], coconut milk [11], sugar manufacture processing waste [12], fermented radish brain [13], corn meal, pea nut meal, soybean meal, coconut residue [14], whey waste [7], corn syrup [15], grape must, beet molasses, soybean flour extract, maize flour extract [16], jack fruit seed [2], plant extract [17], prehydrolysed wood [18] etc. Efforts have been made in order to reduce the production cost of microbial pigments as compared to synthetic, plant and animal derived pigments [16, 19].

This paper outlines the following objectives. 1) Isolation of the pigment producing bacteria from garden soil, 2) Examine the potential of cheap natural substrate like rice powder for the production of pigment, 3) Extraction, purification and analyzing the chemical nature of the produced pigment, 4) Investigating the stability of the obtained pigment, and 5) Optimization of key production parameters, using Taguchi experimental design tool.

4.3.2 Materials and methods

4.3.2.1 Sampling, microscopy and strain characterization

Carotenoid producing microbial cultures was isolated from soil sample collected from the premises of NIT campus Rourkela. Screening procedure adopted was plating of 1 mL (10^{-10}) diluted soil samples on nutrient agar (Himedia Pvt Ltd, India) with inoculation at 25 °C. The orange colored colonies developed on the plates were observed. The isolate showing maximum diameter was propagated on rice plates at 25 °C for 24 h. Characterization and identification of the isolate with pigment production ability was carried out morphologically with optical microscope (Hund-H600). In order to study the morphological characters such as size and shape of the microorganism, various images were captured at different magnifications using SEM (Scanning Electron Microscope) analysis [JEOL JSM-6480LV]. 16s-rDNA characterization was performed on this isolated strain at Xcelris labs Ltd, Ahmedabad, India.

4.3.2.2 Pigment production

Finely ground rice powder was used as a substrate for pigment production. Experiments were conducted in petriplates of 10 cm diameter containing 3 g of rice powder. The substrate was moistened with double distilled water (8 mL) at neutral pH - 7 and autoclaved to maintain sterile conditions throughout the study. On cooling to room temperature; moisture content of the substrate was analyzed using moisture analyzer (Sartorious MA-150) and was estimated as 52.04 %. The substrate in plates was inoculated with 1 mL inoculum culture and incubated at ambient room temperature ~ 30 °C for 10 days. After observing intense coloration in rice plates, batch experiments at pH 6 and 8 were also studied for pigment production.

4.3.2.3 Purification and stability of the pigment

Pigment was extracted using a modified method adapted from Babitha et al. [20]. To evaluate the solubility of the pigment a definite quantity of substrate (rice powder) 1g was extracted with fixed volume 5 mL of different solvents such as acetone, methanol, ethanol, chloroform, water and 10 % NaOH [21]. The pigment-solvent mixture was allowed to stand after shaking for 15 minutes. Based on high extraction of pigment and fair dispersion with 10 % NaOH, ethanol was selected as extracting solvent. About 1:5 rice powder (grams) to solvent (mL) ratio was used for pigment extraction by thorough dispersion as per the reported method [2]. The mixture was centrifuged at 9000 rpm and the supernatant was collected. The above process was repeated until the substrate was colorless. Phase separation of the extracted pigment in the supernatant was achieved on adding equal volumes of diethyl ether and NaCl (0.1 M) to obtain pure pigment devoid of cellular disturbances [11]. The obtained pigment solution was further purified using thin layer chromatography using stationary phase - TLC silica gel 60 F_{254} and hexane: methanol (7:3) as stationary phase and solvent phase respectively. To obtain orange colored powdered pigment the TLC extract was subjected to vacuum evaporation.

The extracted pigment was assessed on 0.50 g of dry pigment and adjusted to 50 ml constant volume of ethanol before measuring its absorbance at (λ_{max}) using a UV-3600 Shimadzu UV-Visible spectrophotometer. The structural analysis of the extracted pigment was examined by FTIR (Bruker ATR-FTIR, USA) at room temperature. Stability of the purified pigment was also tested after thermal treatment at 45 ^{0}C for 8 h at ambient conditions in an oven. It was subjected to UV-visible, fluorescence and Attenuated total reflectance

Fourier transform infrared (FTIR) spectroscopy analyses to observe the structural confirmations.

4.3.2.4 Optimization of key parameters

Taguchi experimental design method was employed to evaluate the optimum conditions for pigment production. The key parameters such as pH, temperature and moisture content were studied. A standard L16 (4^3) with 15 degree of freedom was used to examine three factors in four levels. The levels of the factors studied and the layout of the L16 orthogonal array are represented in Table 4.3.1 and 2. ANOVA technique was adopted to analyze statistically and determine the factors that significantly affect the pigment production. The controlling factors were identified with the magnitude of effect quantified. Consequently, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. All calculations were performed using MINITAB® Release 14.1 software.

4.3.3 Results and discussion

4.3.3.1 Colony Screening, pigment production

Distinct microbial colonies were observed on the agar plate after three weeks of incubation. An orange colored peculiar spot surrounded with cream white color colony was screened and cultured for the pigment production. After two days of incubation at 35 °C, visible cream white color colonies were identified on the agar slants. Incubation for another three weeks resulted in the production of evenly spaced orange-red spots (Fig. 4.3.1a). These colonies were preserved using paraffin method as per the protocol [22]. Previous investigations have revealed the utility of rice as a nutrient supplement in fungal pigment production [9, 23]. In

our study rice powder alone was used as a sole nutrient medium for orange colored pigment production through bacterial species. Using rice powder as nutrient medium an intense orange colored pigment was observed on the plate after 10 days of incubation at ambient temperature of 30 °C (Fig. 4.3.1b). During the incubation, it was noted that the pigment intensity was more on the surface of the rice plate than the deeper layers of the plate. The pigmented rice material was suspended in ethanol for pigment extraction and subjected to further purification steps as ascribed in the above section via thin layer chromatography and was shown in Fig. 4.3.1c,d. At constant temperature (30 °C) and varying the pH (6 and 8) did not show notable color change on rice plates (Fig. 4.3.2).

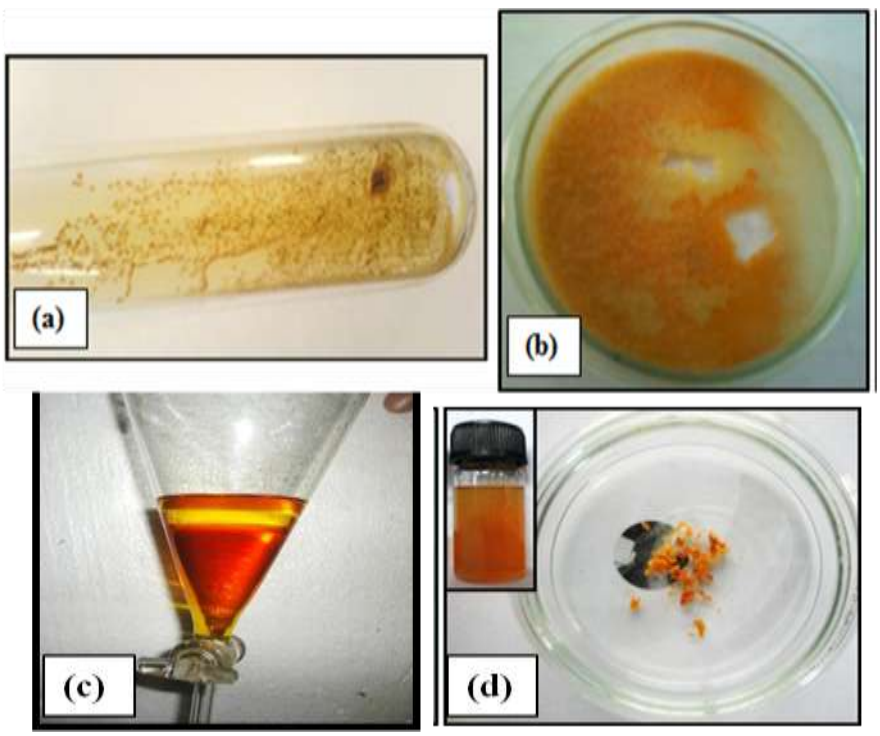

Fig. 4.3.1. Microbial isolate with orange – red spotted colonies on nutrient agar slant (a), and orange pigment by the isolated soil microorganism on rice powder (b). Phase separation of the obtained pigment was shown in (c). Resultant separated pigment concentrate in liquid (d insert) and in solid form after vacuum drying was also given in (d).

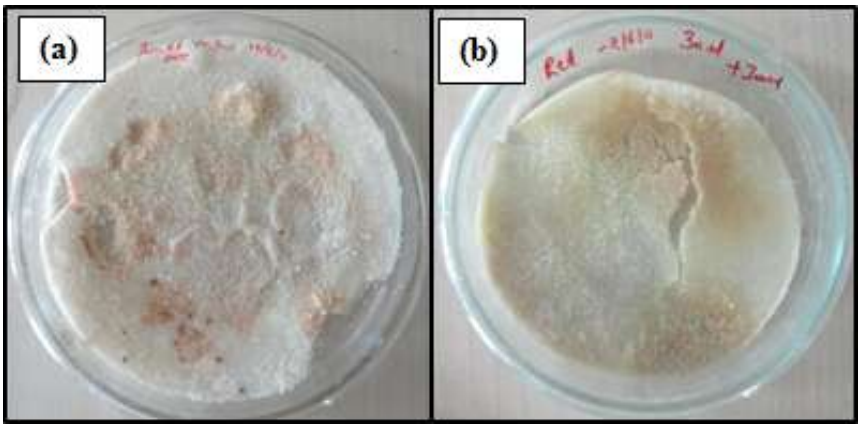

Fig. 4.3.2. Orange pigments by the isolated soil microorganism on rice powder plates at pH - 6 (a) and 8 (b) respectively.

4.3.3.2 Microscopy and microbial characterization

Fig. 4.3.3a shows the optical microscopic image of the microbial isolate and the produced pigment. The nature of the obtained pigment was agglomerative and extracellular. It was also evident from the small size of the microbe involved that the bulky pigment clumps might be their possible metabolites. SEM analysis confined the morphology of the microbe (Fig. 4.3.3b,c). It clearly suggests a rod shaped bacterial species of the size range 1.1 to 1.5 micron. These colored colonies based on morphology and phylogenic analysis through 16s rDNA sequencing were identified as *Bacillus clausii* XJU-3, This bacterium strain showed 100 % homology with *Bacillus clausii* (AY960116.1) and the phylogenic relationship is shown in Fig. 4.3.3d. Reports on production of yellow, orange and pink carotenoids on a variety of substrates suggest that the identified species has potential for carotenoid pigment production [24-25].

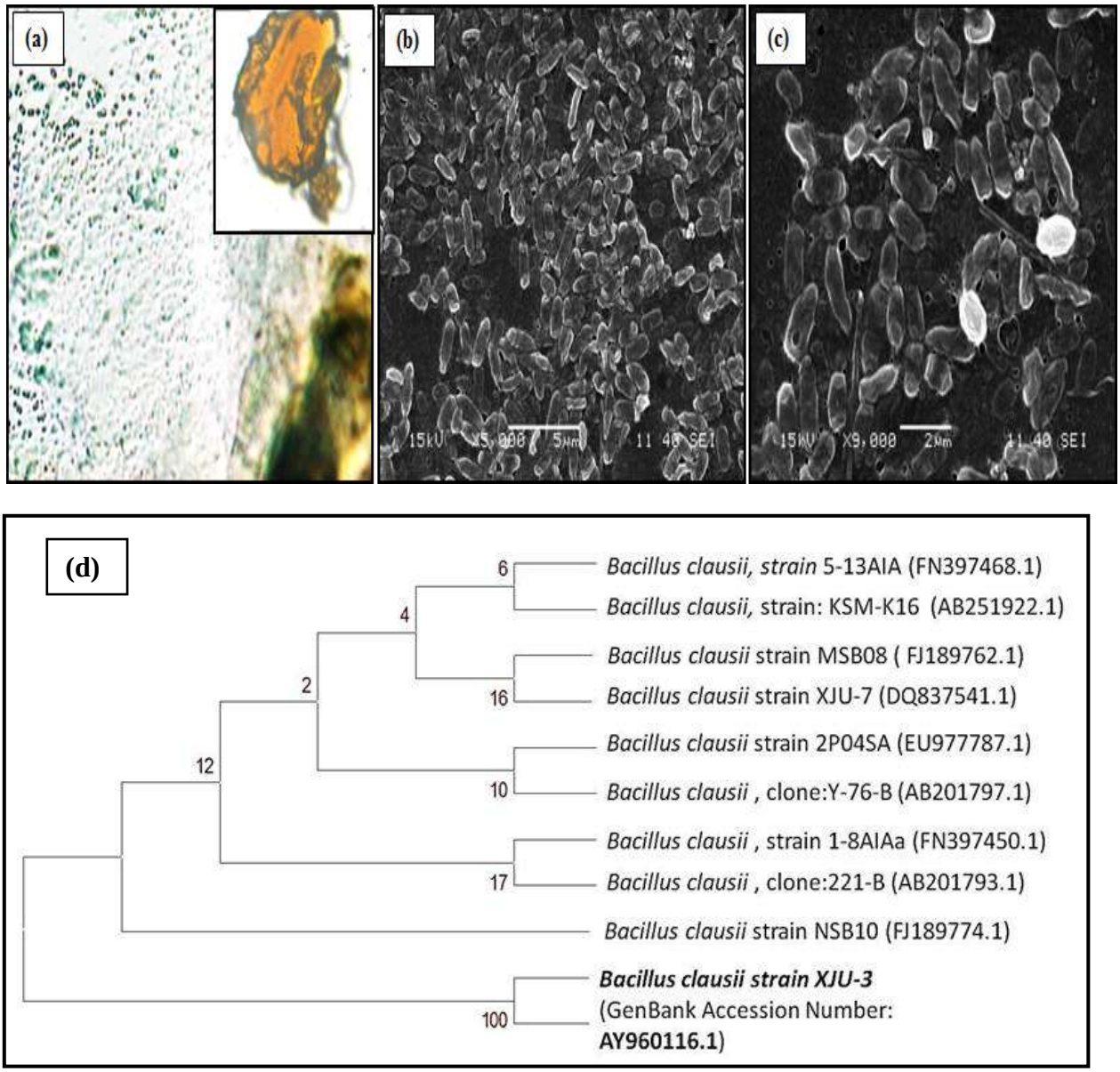

Fig 4.3.3. Pigment producing bacterial species with an orange pigment (insert) magnified by an optical microscope at 400 X magnification (a). SEM images of the identified pigment producing bacteria with (b) 5000 X, (c) 9000 X magnifications and (d) Phylogenetic tree showing the position of the isolate XJU-3 with reference to related strains

4.3.3.3 UV-Visible/fluorescence spectrophotometry and stability of the pigment

Fig. 4.3.4a shows the UV-Visible spectrum of pigment solution (extracted in ethanol) produced from the isolated *B. clausii* on the rice medium. The absorption maxima were observed at 447 and 472 nm and the spectral pattern were in close resemblance to the characteristic of β-carotene [26]. Literature shows that β-carotene type carotenoids are sensitive to oxidation, light and heat [27]. To confirm the pigment kind, pigment stability under different storage conditions was tested. In general, β-carotene is stored in aluminum foil bags under inert gas atmosphere at low temperatures < 15 ^{0}C to retain > 96% of the activity [28]. To test the degradability and stability of the observed pigment it was subjected to thermal drying under aerobic conditions in a closed oven for 8 h at 45 ^{0}C. After incubation, the color of the pigment residue became slightly shaded from orange-red to pale yellow, which may be due to oxidation of the pigment. It was subjected to further analyses (UV/fluorescence).

Fig. 4.3.4b shows the spectrum of the shaded pigment and the discrete difference in spectra of (a) and (b) might be due to oxidation of the produced pigment. The band maximum of spectrum in (b) was found to be at 330 nm with a shoulder peak at 345 nm, while for the diluted solution of the same, an additional absorption at 262 nm was observed (Fig. 4.3.4b insert). The absorption of spectrum (b) at 330 nm of the electromagnetic spectrum indicates close spectral resemblance of vitamin A class retinol pigment [29]. The additional peak split at ~ 340 nm and band at 262 nm specify that the extracted pigment was in its oxidized form [30]. An interesting feature of these retinol class compounds absorption at shorter wavelength region is due to their highly twisted double-bond in their systems [31]. The above evidence shows that the nature of the pigment obtained from *B. clausii* is of wholesome β-carotene and

produced products like precursors of vitamin A as a result of aerobic thermal oxidation, which usually occur via chemical transformation [32]. The evaluation of fluorescence property of the oxidized pigment also confirms the property of retinol type products [32]. The maximum excitation was found to be at 315 nm and the λ_{emi} is observed at 382 nm as shown in Fig. 4.3.4c. The fluorescence yield of pigment before thermal treatment has produced no or negligible spectral signal and was not reported.

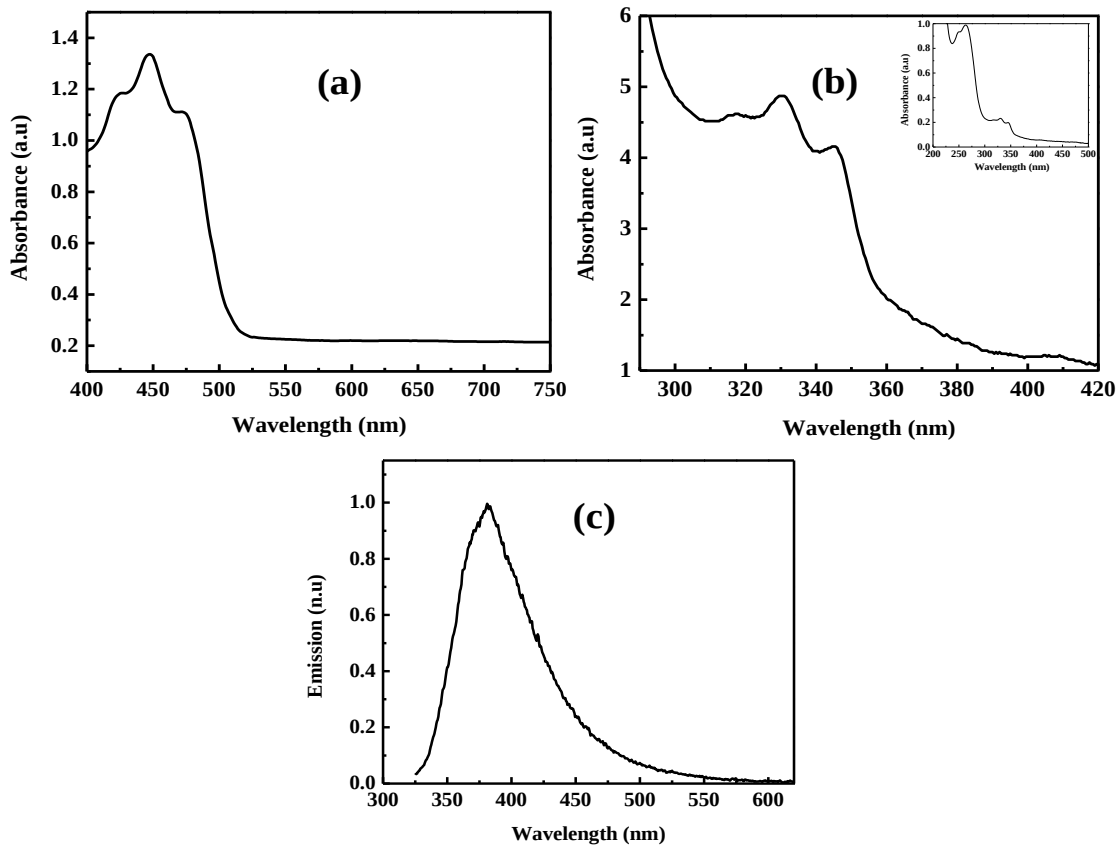

Fig. 4.3.4. (a) UV- visible spectrum of the extracted orange pigment in ethanol, (b) UV absorption of the oxidized pigment with narrow and wide (insert of (b)) spectral regions, and (c) Fluorescence emission spectra of the oxidized pigment at 382 nm.

4.3.3.4 FTIR analysis

In order to understand the pigment structure FTIR analysis was performed. The purified pigment was subjected to FTIR spectroscopy before and after oxidation steps (Fig. 4.3.5a,b). Fig. 4.3.5a shows the IR spectrum of the pigment with major peaks at 3320, 2943, 2832, 1652, 1448, 1405, 1100 and 1020 cm^{-1}. The majority of the peaks from the obtained pigment show a close resemblance to a β-carotene structure [33-34]. Peaks at 2943 and 2832 cm^{-1} are due to asymmetric and symmetric stretching vibrations of CH_2 and CH_3 groups [34]. And peaks at 1020 cm^{-1} is for wagging vibration of (RH).C=C.(RH) groups of the synthesized pigment. Peaks at 1405 and 1448 cm^{-1} are due to symmetric deformation of δ CH_3 and deformation vibration of δ CH_2 groups. Moreover the peaks at 3320, 1652 and 1020 cm^{-1} are due to vibrational modes of water interference in the analyzed pigment [33, 35].

Fig. 4.3.5b shows FTIR peaks after the oxidation of the pigment. The major peaks observed here are at 3433, 3229, 3000, 2883, 1672, 1388, 1343, 1089, 1082, 884, 708 and 631 cm^{-1}. The spectral pattern highly resembles retinol compound of carotenoids family [36]. The absorptions in the region of 3200 - 2700 cm^{-1} are normally characteristic of carbon and hydrogen containing species, and can be assigned to various forms of C-H stretching. The absorbance at and above 3000 cm^{-1} suggest that the compound is likely to have unsaturated C=C. The absorption at 3433 cm^{-1} which is in the region of 3650-3250 cm^{-1} mainly occurs for hydroxy or amino groups.

The additional intense bands in 1600-1300, 1200-1000 and 800-600 cm^{-1} in Fig. 4.3.5b confirms that the oxidized compound consists a simple hydroxy compound with O-H absorption and is likely to be an alcohol. The absorption at the low end and below 1700 cm^{-1} was due to carboxylate (carboxylic acid salt). In our case the peak at 1672 cm^{-1} may be due to

conjugation at C=O. (may be our oxidized compound is a mixture of retinol and retinoic acid). Peaks at 3000 cm^{-1} and 2986 cm^{-1} which are just above and below 3000 cm^{-1} may be due to unsaturated and saturated C-H absorptions in the compound. Bands at ~ 1000 cm^{-1} and 880 cm^{-1} indicate C-H out of plane bending which is usually seen for all *trans* retinol compounds. Further the olefinic double bond type unsaturation can be confirmed by a single peak around ~ 890 cm^{-1}along with the absorptions at ~1650 and ~3000 cm^{-1} [35]. The structural confirmations by IR analysis in concurrence with UV-Visible analysis infer that the produced pigment is exclusively close to β-carotene in all aspects. And the retinol derivatives of the obtained pigment on thermal treatment further confirm the instability of these β-carotene type pigments.

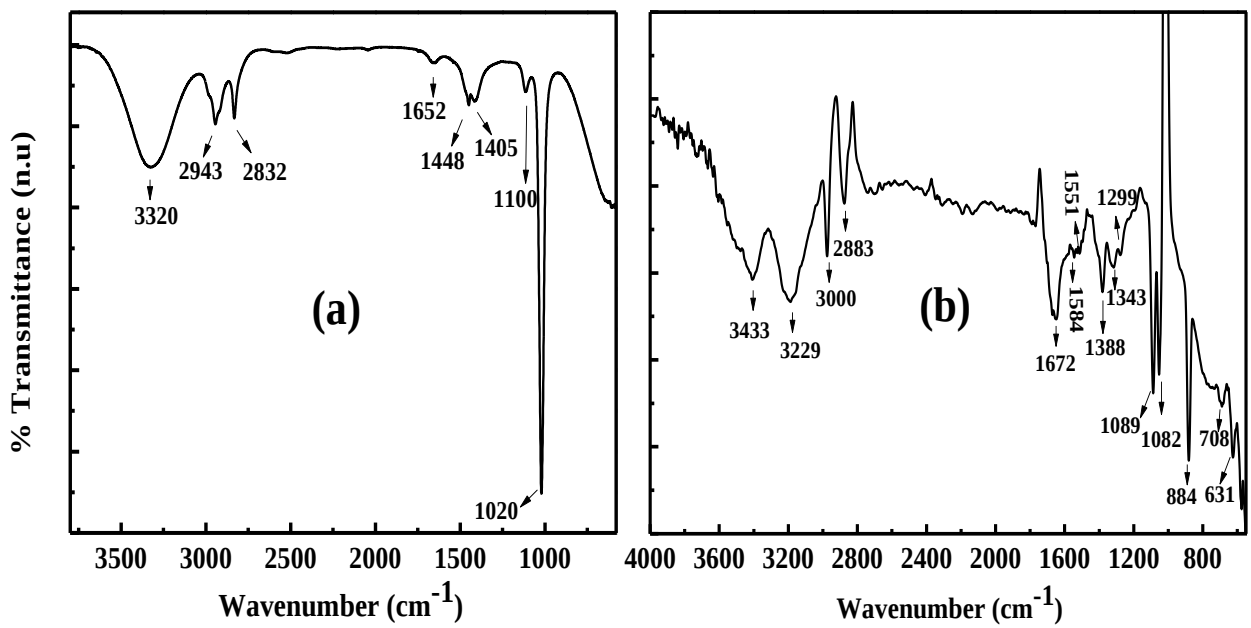

Fig. 4.3.5. ATR-FTIR analysis of the extracted and purified orange pigment before (a) and after (b) oxidation process.

4.3.3.5 Optimization of key production parameters

Extensive research focused on the optimization of the key process parameters for high yield carotenoid production by employing simple statistical procedures [37-38]. The above analysis inferred that the pigment produced by the bacterial isolate *B. clausii* needs proper attention while purification and preservation as it is thermally sensitive at normal atmospheric conditions. By following cautious steps and to know the influential factors for the pigment production in higher amounts; effect of key parameters like pH, temperature and moisture are studied by using a simplistic Taguchi method. This method of optimization offers ease in identifying the key process parameters and is a fractional factorial experimental design. This method can be effectively used in optimization of biotechnological processes, since it has the ability to include categorical factors also [39].

The results of the experiment showed that the maximum yield of pigment was 95.8 mg/3 g rice powder, at optimum experimental condition (pH 7, temperature 30 °C and moisture 55 %) (Table 4.3.2). Fig.4.3.6 depicts the main effect of each factor (Table 4.3.2), he term 'main effects' considered as average of the obtained results in which each factor is at a given level. Results obtained using ANOVA software (Table 4.3.3) suggests the significance of the factor in the following order pH > temp with p - values of 0.011 and 0.065. Thus these p-values (on comparison with α – level values) indicate that the parameter pH and temperature are significant at the levels 0.05 and 0.010 α respectively and are significantly related to the response. The average yield of the pigment was estimated to be 69.4 mg/1g rice powder (Table 4.3.2). Table 4.3.4 shows the suggested conditions as per calculations. The statistical calculations as per the conditions suggested in Table 4.3.4 predicted that if the conditions were chosen as shown in Table 4.3.4, should produce 103.3

mg pigment per 1 g rice powder which is 48.9 % more yield. However, the experimental result showed 107 ± 1.2 mg pigment production using 1 g rice powder since the deviation in actual and predicted value was only about 3.58 %, which is acceptable. Therefore on comparing with 95.8 mg pigment produced before, a further increase of about 11.6 % was achieved.

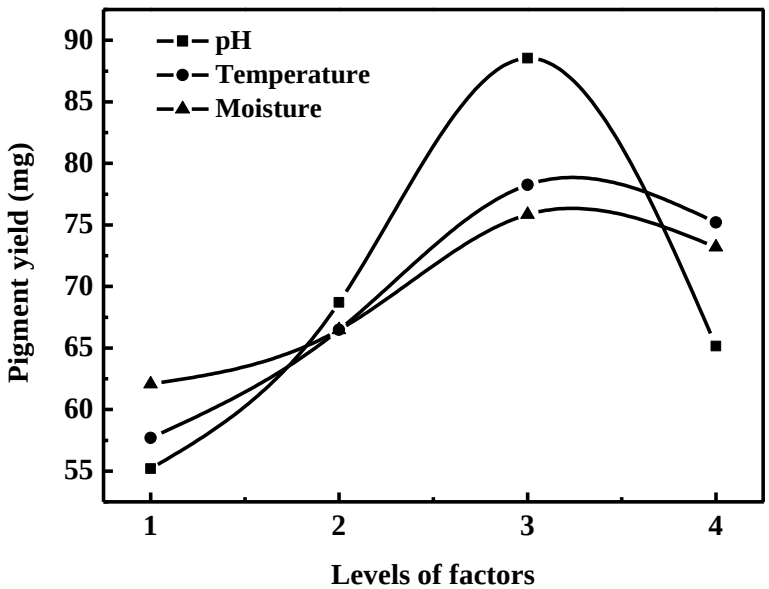

Fig. 4.3.6. Main effects of factors or average of obtained results (pigment per 3 gm rice powder) in which each factor is at a given level. For detail about 'levels' refer to Table 4.3.1.

Table 4.3.1. Factors and their levels studied by Taguchi method.

Factor	*Level 1*	*Level 2*	*Level 3*	*Level 4*
pH	6	6.5	7	7.5
Temperature (^{o}C)	25	30	35	40
Moisture % (w/w)	40	45	50	55

Table 4.3.2. Levels of three different factors in each of sixteen trails and obtained results.

pH	*Temperature*	*Moisture*	*Pigment yield - mg/3 g substrate*
1	1	1	46.1
1	2	2	52.4
1	3	3	68.3
1	4	4	54.0
2	1	2	50.2
2	2	1	49.3
2	3	4	86.6
2	4	3	88.7
3	1	3	78.0
3	2	4	95.8
3	3	1	87.6
3	4	2	92.8
4	1	4	56.4
4	2	3	68.4
4	3	2	70.5
4	4	1	65.3

Table 4.3.3. Analysis of variance of main effects of factors. SS/MS indicate sum/mean of squares in the table.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	3	2347.7	2347.7	782.55	9.46	0.011
Temperature	3	1032.0	1032.0	343.99	4.16	0.065
Moisture	3	473.0	473.0	157.67	1.91	0.230
Residual error	6	496.3	496.3	82.72		
Total	15	4349.0				

Table 4.3.4. Optimum conditions suggested by statistical analysis after performing the tests

Factor	*Level description*	*Level*	*Contribution greater than avg. yield in 'mg' - in '%'.*
pH	7	3	19.10 – 27.0
Temperature (^{o}C)	35	3	8.85 – 12.7
Moisture % (w/w)	50	3	6.45 – 9.2

4.3.4 Conclusions

The production of orange pigment showed promising results using a soil microorganism with rice powder as a sole substrate. Microscopic studies exposed the morphological characteristics and 16S rRNA sequence analysis confirmed the bacterial isolate as *B. clausii*. Observations by UV– visible absorption spectrum and FTIR analysis of the purified pigment showed it's resemble to the carotenoid class compound β-carotene. The thermal sensitivity of the obtained orange pigment was well explored by UV, fluorescence, FTIR analyses and the residual products were structurally close to β-carotene derivatives like retinol and retinoic acid. Maximum pigment production was achieved (107 ± 1.2 mg/1 g rice powder) by Taguchi method of optimization at pH - 7 and 35 ^{o}C temperature. The high sensitivity in yield with respect to close variation in key parameters (like temperature and pH) was studied.

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Chapter 4 Approach and investigations

Part II - Facile Synthesis of Commercial Pigments by Purchased Strains on Cheaper Substrates

4.4 Carotenoid by *Rhodotorula sp.* on Fruit Waste Extract as a Sole Carbon Source and Optimization of key production parameters

4.4.1 Introduction

Pigments are of great commercial interest and have received considerable attention because of their potential beneficial effects on human health besides pigmenting properties [1]. In recent years there is an increasing demand for microbial pigments as promising alternatives

for synthetic pigments that are widely used in food industries [2]. The pigments from microorganisms are nature selected and have advantages over plant and animal derived pigments with no seasonal variations, no geographic inconsistency in the production, high productivity and are easily manipulated in the processing schemes [3-4].

Among microbial derived pigments, carotenoids are fat soluble diverse class of yellow, orange, red and purple natural pigments which are unanimously produced by a wide range of microorganisms and plants. Due to the recent discovery of anticancer and antioxidant properties of carotenoid pigments, their use in pharmaceuticals and neutraceuticals is expected along with their applications in the fields of food, cosmetics, chemicals and so on [5-7]. Globally carotenoids are estimated to supersede USD S 280 million in 2015 [8]. In order to improve the yield of carotenoid pigments and to decrease the cost of production, various studies have been performed using several microorganisms using numerous different substrates [9-10]. Amongst different microorganisms *Rhodotorula sp*. stood as a potential and scientifically favorable candidate for high yield carotenoids production [11].The yeast *Rhodotorula sp.* is one of very few types of yeast which is able to produce a large number of carotenoid pigments which are mainly β - carotene, torulene, torularhodin [12]. Moreover, many investigations have been done using this genus to make the process economical [3, 6, 13-14]. Key process parameters like type of substrate, nutrients availability, pH, temperature and so on are optimized in various processes [15]. However, there are still extensive investigations focused on this versatile genus *Rhodotorula* to produce carotenoids in more economical ways.

The cost of the substrate has an important contribution to the overall pigment production cost, and it can be minimized by using cheaper organic waste. Substrate

composition, typically carbon source has a significant effect on carotenoid production and its cost. Many substrates have been considered as potential substrates for carotenoid production [16]. Using a low cost or cheap substrate for the production of high value products may help to make the process economical. Till now, several cheaper substrates have been used (e.g. sugar cane juice, peat extract, whey, grape must, beet molasses, hydrolyzed mug bean waste flour, soyabean and corn flour extract and sugar cane molasses etc.) for the production of carotenoids [17]. In this study, we have used fruit waste extract (FWE) as the substrate for the production of carotenoids. In general, fruit processing units dispose the wastes which are rich in soluble sugars and micronutrients that support the microbial growth. Nowadays, fruit waste disposal is one of the problems that the fruit processing industries are facing. Eyeing on this, we have chosen fruit waste extract as potential substrate for pigment production which can reduce the production cost and make the product economical. Using FWE as a suitable sole substrate; we examined the ability of *Rhodotorula rubra* for carotenoids production.

In recent years statistical design has been successfully employed to identify the optimum level of various parameters involved in the process. Statistical design is a powerful tool that accounts for the main as well as interactive influences of the parameters on the process performance. The disadvantages of other classical methods are that they are time consuming, laborious and expensive. In contrast, the use of statistical tools such as RSM methodology provides a great amount of information based on only a small number of experiments [18]. In this study a combination of traditional non statistical and statistical method based experimental design has been employed to optimize the biomass and carotenoid production. Primarily, one factor at a time, a classical approach was practiced that involves various levels of one factor when the other factors are constant. Using this method,

the key parameters which influenced the pigment production significantly, were identified. Box–Behnken experimental design method is useful for rapidly optimizing the process with limited number of experiments. Hence the objective of this study is to explore the effectiveness of the sole substrate (mixed fruit extract) on pigment production using the Box-Behnken statistical tool. Optimization of process parameters such as pH, temperature, and agitation for high yield of biomass and pigment production was experimented.

4.4.2 Experimental section

4.4.2.1 Reagents and chemicals

Methanol, acetone, HCL, NaCl, and diethyl ether were procured from Merck, Mumbai, India., malt yeast extract medium, malt yeast extract agar were purchased from HiMedia, India. All other chemicals used were of analytical reagent grade throughout the study. Double distilled water was used through the study and aseptic conditions were maintained wherever necessary.

4.4.2.2 Microorganism and its maintenance

The microorganism *Rhodotorula rubra* (MTCC no: 1446) used in this study was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The microorganism was grown and maintained on malt yeast extract medium and stock cultures were preserved on malt yeast extract agar slants at 4 ^{0}C and sub-cultured at monthly intervals.

4.4.2.3 Substrate preparation

Fruit waste typically containing pineapple, pomegranate and orange is obtained from a fruit juice shop of a local market. It includes extracted carpels of oranges, core of pineapples, and crushed seeds along with arils of pomegranate. The soluble sugars are extracted from 1

kilogram of fruit waste by adding 2 liters of distilled water at 100 ^{0}C for 30 minutes. The resultant straw colored fruit waste extract (FWE) is filtered and stored at 4 ^{0}C for further experimentation.

4.4.2.4 Pigment production and extraction

The biomass (5 mg) was activated in malt yeast extract broth (100 ml) at 30 ^{0}C for 24 hrs. 1ml of the activated culture was inoculated in 250 ml conical flasks containing 50 mL autoclaved FWE medium maintained at different pH's (4.3, 5, 6, 7, 8). Until significant color appearance the incubation at 30 ^{O}C was continued. The pigmented biomass collected after centrifugation (9000 rpm, 10 min) was washed repeatedly (thrice) with distilled water and subsequently treated with 1 N HCl (60 ^{0}C, 10 min).

The treated cells were further washed in sterile distilled water and subjected to repeated solvent washes to extract the intracellular pigment. Methanol: acetone (1:1) was used as solvent for the microbial pellet wash and washing step continued till colorless pellet was achieved. The washed colored solvent was collected separately and extracted with equal parts of diethyl ether and NaCl (10 %) as a part of further purification step of the carotenoid pigment. The obtained pigment here was subjected to thin layer chromatographic purification step as stated in Chapter 4.3. The total carotenoid concentration (measured as β - carotene) in diethyl ether extract was determined using standard method [13]. It was estimated according to the absorbance at 448 nm using spectrophotometer (UV-3600 Shimadzu). The pigment quantity is estimated using absorption coefficient $E^{1\%}_{1cm}$ = 2659 via spectral analysis. To obtain purified pigment in solid form, the collected ether layer subjected to vacuum drying.

4.4.2.5 DPPH assay

The effect of carotenoid form *R. rubra* on DPPH free radical was studied following the method of [19] with some modifications. 0.1 mM DPPH solution in ethanol (99.5 %) was prepared. Pigment in ethanol (1.65 mg/ml) in different volumes (10, 20, 30, 50 and 70 µl) was made up to 100 µl with ethanol and was added to 1 ml DPPH solution. The mixture was thoroughly mixed and allowed to stand for 40 min at room temperature in the dark. The decrease in the absorbance at 517 nm was then measured. Ascorbic acid is used as positive control and the pigment solution without DPPH as negative control. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following formula:

$$\%\ \text{Scavenging acticity} = \frac{(\text{A}c - \text{A}s)}{(\text{A}c)} \times 100$$

Where A_c,A_s are absorbance's from control and sample.

4.4.2.6 Analysis

Cell dry weight was measured by harvesting the cells after centrifugation of the growth medium at 5000 rpm for 10 minutes and subsequent washing thrice with distilled water. The cells were dried at 105 ^{0}C till constant weight was attained. Reducing sugars were estimated during experimentation by DNS assay method [20]. Elemental analyses were determined on a Vario EL Cube CHNS Analyzer for FWE, before and after carotenoid production at pH -7.

4.4.2.7 Experimental design

One factor at a time and Box-Behnken design were used for optimization of carotenoid production. The significant effect of pH towards carotenoid and biomass yield was determined by one factor at a time method. Knowing the safe operating zones of other

production parameters like temperature and agitation, a Box-Behnken design (BBD) was selected for optimization of the mentioned key process parameters (Table 4.4.1) for carotenoids production by *R. rubra*.

Table 4.4.1. Coded and actual levels of the three variables

Variable	Symbol	Coded and actual levels		
		-1	0	1
pH	x1	6	6.5	7
Temperature	x2	25	27.5	30
agitation	x3	90	120	250

Table 4.4.2. Experimental design matrix for the Box-Behnken design.

Run	pH (x1)	Temperature (x2)	Agitation (x3)	Biomass-mg/ml	Pigment-mg/l
1	0	-	-	3.33	1.33
2	0	-	+	4.01	1.74
3	+	0	-	5.3	2.12
4	+	-	0	6.31	2.51
5	+	+	0	7.21	2.77
6	-	-	0	2.74	1.09
7	0	0	0	6.44	2.47
8	0	0	0	6.76	2.71
9	-	0	+	4.99	1.98
10	0	0	0	6.54	2.62
11	-	+	0	5.2	2.09
12	+	0	+	7.75	3.1
13	-	0	-	3.87	1.55
14	0	+	+	7.08	2.72
15	0	+	-	4.8	1.92

The experimental design and statistical analysis of the data were carried out using the Minitab (14.0) statistical software package. BBD with three factors and 15 runs was chosen; a model was developed and also validated. Table 4.4.1 shows the variables and experimental design levels for response surface and Table 4.4.2 shows the design matrix of BBD.

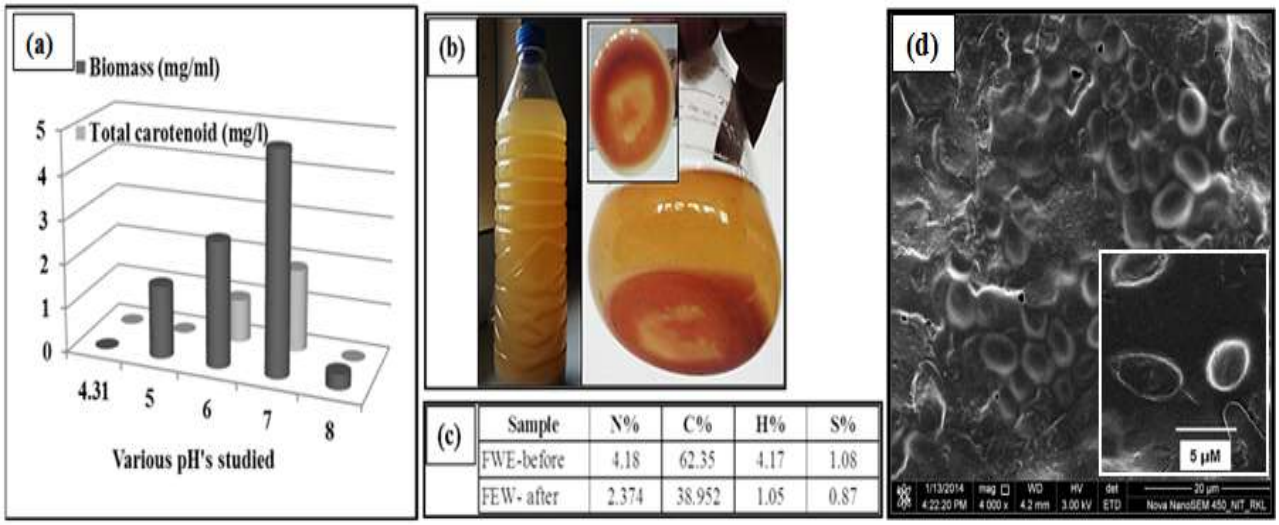
4.4.3 Results and discussion

4.4.3.1 Effect of pH on biomass and carotenoid yield

Using first approach the significant parameters that affected carotenoid production were identified as pH, temperature and agitation. Effect of pH on biomass growth and carotenoid production is illustrated in Fig. 4.4.1(a). The figure shows native FWE's pH (4.31) including different values studied. For evaluating the influence of pH on biomass and pigment production, *R. rubra* was cultivated in FWE medium at 30 ^{0}C for 7 days. The microbial culture is inoculated in the medium with adjusted pH's 5, 6, 7, 8, and 9 along with initial pH 4.31. Among various pHs, biomass growth was identified at values of 5 to 9 and pigment production was observed only at 6 and 7. So, the results suggest that pigment production was highly dependent on pH irrespective of biomass growth with high yield caused at pH 7. The prepared FWE and fermented FWE with the yeast *R. rubra* are shown in Fig. 4.4.1(b).

The reducing sugar content in FWE was identified to be 19.90 mg/ml before experimentation. CHNS analysis was performed for the prepared FWE so as to estimate the presence of major and minor nutrients. CHNS analysis was done before and after carotenoid production (Fig. 4.4.1(c)). The values stated suggests that significant utilization of macro and micro nutrients by the microorganism followed the following order of H > N > C > S. In

addition to the above, near and far view SEM image of the used yeast was also shown in Fig. 4.4.1(d) and its insert.



Sample	N%	C%	H%	S%
FWE-before	4.18	62.35	4.17	1.08
FEW- after	2.374	38.952	1.05	0.87

Fig. 4.4.1. (a) Growth of biomass and carotenoid yield at various pHs and at room temperature (30 ^{0}C). Prepared fruit waste extract (FWE) – left, and FWE with *R. rubra* growth with intracellular carotenoid (right) upon incubation (b). CHNS analysis of FWE before and after fermentation; upon separation of *R. rubra* by centrifugation (c). SEM image of the used yeast.

4.4.3.2 Biomass growth, pigment yield and glucose utilization

Fig. 4.4.2A presents the individual values of cell dry weight (mg/ml), total carotenoid content (mg/l) and glucose concentration (mg/ml) as a function of time in days. Pattern from the figure shows that carotenoid content paralleled the cell dry weight and substrate concentration decreased linearly till 3 days (72 h). Fig. 4.4.2A represents the growth of biomass and production of carotenoid with time. Initially the rate of growth of biomass superseded the carotenoid production. However after 3 days the biomass yield parallel to

carotenoid production rate. On the contrary the substrate utilization rate also diminished after 3 days. This graphical representation suggests that maximum carotenoid production was achieved at the end of log phase at the expense of substrate utilization. Thereafter with the approaching stationary phase, the carotenoid production capacity was constant which is typical of any production process in a batch reactor. The observed condition shows the biomass's pigment production ability irrespective of quantitative substrate utilization. However maximum pigment can be produced within 4 days of incubation using FWE as sole substrate.

The UV-visible spectrum of the pigment produced was illustrated in Fig. 4.4.2B. The observed absorption maximum at 507 nm towards the higher wavelength states that the produced compound resembles Torularhodin type carotenoid, which is usually produced by *Rhodotorula* in significant quantities [5]. The conformation study showing structural details using FTIR analysis was carried out on the produced pigment along with the reference β - carotene (Fig. 4.4.2C-a, b).

The FTIR spectrum of extracted carotenoid is shown in the Fig. 4.4.2C-a. The peak descriptions can be described [21] as follows: the broad peak at 3351 cm^{-1} (which is in between 2900 to 3500 cm^{-1}) is due to hydrogen bonded O-H; peak at 2873 cm^{-1} is due to methyne C-H stretch; peaks at 1692, 1636 cm^{-1} is for alkenyl C=C and aryl substituted C=C in the compound; peak at 1429 cm^{-1} attribute to methyl C-H asymmetric band; peak at 1231 cm^{-1} is for C-O stretch and 1081 cm^{-1} is for C-C skeletal vibrations. The peak bands of 3351 along with peak at 1231 cm^{-1} confirms the presence of carboxylic group in the purified carotenoid.

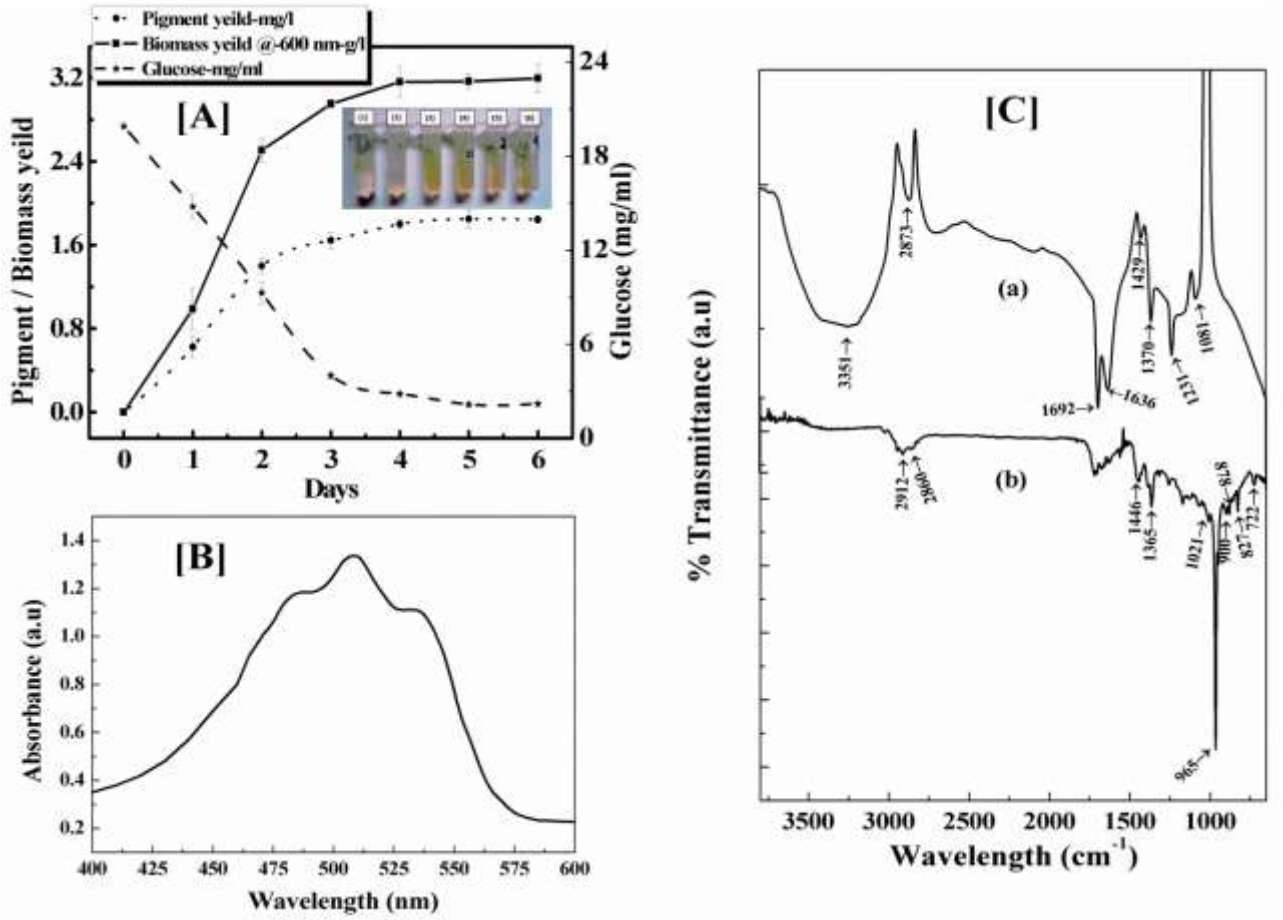


Fig. 4.4.2. Carotenoid production (mg/l) and biomass yield (mg/ml) along with glucose utilization (mg/ml) given in [A]. The picture insert shows the colored biomass from 1 to 6 days. UV-visible spectrum of the purified carotenoid in methanol medium [B]. FTIR spectra of the synthesized carotenoid (a) and the purchased β-carotene (b) pigment.

Moreover on comparison with the purchased β - carotene's spectrum (Fig. 4.4.2C-b), the obtained carotenoid spectrum has some similarities and some variations. The spectrum of β-carotene displayed major peaks at 2912 and 2860 cm^{-1} (for asymmetric and symmetric vibrations of the CH_2 and CH_3); peak at 1446 cm^{-1} (due to CH_2 group); peak at 1365 cm^{-1} (for splitting due to dimethyl; group); peaks at 1021 cm^{-1} (is for in plane –CH-) and 965 cm^{-1} (is

for trans conjugated alkene -CH=CH- out-of-plane deformation). Further peaks at 700 to 900 cm^{-1} are for skeletal vibration of C-C stretch. The IR analysis along with UV spectrum of the obtained carotenoid closely resembles the structures of torularhodin [14] foremost, and has some similarities with β-carotene (as shown in Fig. 4.4.2C-b). Also this spectral pattern is obvious as the mentioned pigments are predominantly obtained by *Rhodotoula sp* [5].

4.4.3.3 Box–Behnken design

The influence of pH on pigment productivity was identified as described in the above section, this indicates a slight deviation of pH values from 6 to 7 resulted in negative effects with low/negligible pigment yield. The above synthesis conditions were at static temperature 30 ^{0}C and agitation (100 rpm). The Box–Behnken design was employed to study the interactions among the significant factors and also determine their optimal levels. This methodology is essentially an assortment of statistical and regression techniques. The first step involves framing a statistically significant empirical model capable of describing the effect of multiple factors on a response. A commonly used empirical model of the response surface analysis is a quadratic polynomial of the type:

$$y = b_0 + \sum_i (b_i x_i) + \sum_i \left(b_{ii} x_i^2\right) + \sum_i \quad \sum_j \left(b_{ij} x_i x_j\right) \qquad (1)$$

where y is the predicted response, $x_i x_j$ are input variables which influence the response variable y; b_0 is the offset term; b_i is the i^{th} linear coefficient; b_{ii} the i^{th} quadratic coefficient and b_{ij} is the ij^{th} interaction coefficient.

Once a suitable model is obtained, it can be used for optimization which involves finding an optimum combination of factors that will maximize or minimize a response. The average yields of biomass and pigment are found to be 5.488 mg/ml and 2.181 mg/l from Table 4.4.2. By using multiple regression analysis, the experimental responses shown in Table 4.4.2 were correlated with two significant factors according to Equation (1).

$$\text{Biomass } ({}^{\text{mg}}/_{\text{ml}}) = 6.58 + 1.2213(x_1) + 0.9875(x_2) + 0.8162(x_3) - 0.9438(x_2 * x_2) - 0.8313(x_3 * x_3) - 0.39(x_1 * x_2) + 0.3325(x_1 * x_3) + 0.4(x_2 * x_3) - \quad (2)$$

$$\text{Carotenoid production (mg/l)} = 2.6 + 0.4737(x_1) + 0.3537(x_2) + 0.3275(x_3) - 0.3725(x_2 * x_2) - 0.3(x_3 * x_3) - 0.185(x_1 * x_2) + 0.1375(x_1 * x_3) - \quad (3)$$

The factors x_1 and x_2 are indicated in their coded units (shown in Table 4.4.1). The goodness of fit of the quadratic polynomials is expressed by the coefficient of determination (R^2- which is a measure of how well the model can be made to fit the raw data). The closer the value of R^2 is to 1, the better is the correlation among the observed and predicted values. The R^2 values for Equations (2) and (3) are 0.956 and 0.960 respectively; indicating that about ~ 95 % of the variations in biomass and carotenoid yield can be explained by the quadratic polynomials. This means that Equations (2) and (3) are adequate for correlating the experimental results. Moreover regression equations (2 and 3) were evaluated by the F-test for analysis of variance (ANOVA). Statistical significance and responses for biomass and pigment production are shown in Table 4.4.3 and 4.4.4. Prob > F value for the model is less than 0.05 infers that the model terms are statistically significant.

Table 4.4.3. Statistical significance obtained for the regression coefficients in Eq's .(1), (2).

Model term	*For Biomass*		*For Pigment*	
	Coef	*p*	Coef	*p*
Intercept	6.5800	0.000	2.6000	0.000
x1	1.2213	0.000	0.4737	0.000
x2	0.9875	0.000	0.3537	0.000
x3	0.8162	0.001	0.3275	0.000
x1*x1	-0.2713	0.166	-0.1125	0.122
x2*x2	-0.9438	0.002	-0.3725	0.002
x3*x3	-0.8313	0.004	-0.3000	0.004
x1*x2	-0.3900	0.059	-0.1850	0.024
x1*x3	0.3325	0.093	0.1375	0.064
x2*x3	0.4000	0.055	0.0975	0.154
	R-Sq = 98.4% R-Sq(adj) = 95.6%		R-Sq = 98.6% R-Sq(adj) = 96.0%	

Table 4.4.4. ANOVA results for biomass and pigment yields.

ANOVA					
For Biomass-mg/ml					
Source	DF	Sum of squares	Mean square	F	P
Regression	9	32.2579	3.58421	34.75	0.001
Linear	3	25.063	8.35433	81	0
Square	3	5.5043	1.83477	17.79	0.004
Interaction	3	1.6906	0.56354	5.46	0.049
Residual Error	5	0.5157	0.10314		
Lack-of-Fit	3	0.4621	0.15403	5.75	0.152

Pure Error	2	0.0536	0.0268		
Total	14	32.7736			
For Pigment-mg/l					
Source	DF	Sum of squares	Mean square	F	P
Regression	9	4.70655	0.52295	38.78	0
Linear	3	3.65468	1.21823	90.34	0
Square	3	0.80132	0.26711	19.81	0.003
Interaction	3	0.25055	0.08352	6.19	0.039
Residual Error	5	0.06742	0.01348		
Lack-of-Fit	3	0.03802	0.01267	0.86	0.576
Pure Error	2	0.0294	0.0147		
Total	14	4.77397			

The actual and predicted values of responses for biomass and carotenoid concentrations versus the corresponding values calculated by regression models are shown in Fig. 4.4.3a,b respectively. Actual values are the measured values for a particular experiment, whereas predicted values are generated by using the approximating functions. The line of perfect fit is also shown in these figures and visualization of the two regression models provides an accurate description of the experimental data. In addition the values of R^2 and adjusted R^2 (Table 4.4.3) have advocated a high correlation between actual and predicted values. The response surface and contour plots were constructed using the regression models and represented in Fig. 4.4.4a-d. Fig. 4.4.4a depicts the interactive effect of the agitation and

temperature on the biomass concentration. At low to moderate agitation values, increases in temperature led to increased production of biomass initially up to center point and then decreased. At low to moderate temperature values, increases in agitation values showed increase in biomass yield. Similar trends were not observed for pigment yield as a function of pH and temperature (Fig. 4.4.4c). At high pH values, increases in temperature showed significant increase in pigment yield up to the center point and decreased thereafter. While with increase in temperature values, increase in pH led to increased pigment production.

The contour plots in Fig. 4.4.4b,c indicate that a local optimum exists in the area experimentally studied; a set of values on the two factors that leads to maximum biomass or pigment production. The location of these optimal points can be obtained by differentiating Equations (2) and (3). Equation (3) was used to derive the most efficient combination of x_1 to x_3 to produce carotenoids from *Rhodotura sp*. According to Equation (3), a maximum carotenoid recovery of 3.17 mg/l could be attained with pH (7.0), temperature (28.2 ^{0}C), and agitation (150 rpm). Experimental validation of the optimum x_1-x_3 combination gave a carotenoid yield of 3.298 ± 0.28 mg/l (with 7.83 mg/ml biomass concentration). Equation (2) depicted that at pH (7.0), temperature (30 ^{0}C), and agitation (150 rpm) maximum yield of 7.97 mg/ml biomass could be achieved. The experimental validation resulted in biomass yield of 8.27 ± 0.33 mg/ml with 2.43 mg/l carotenoid production.

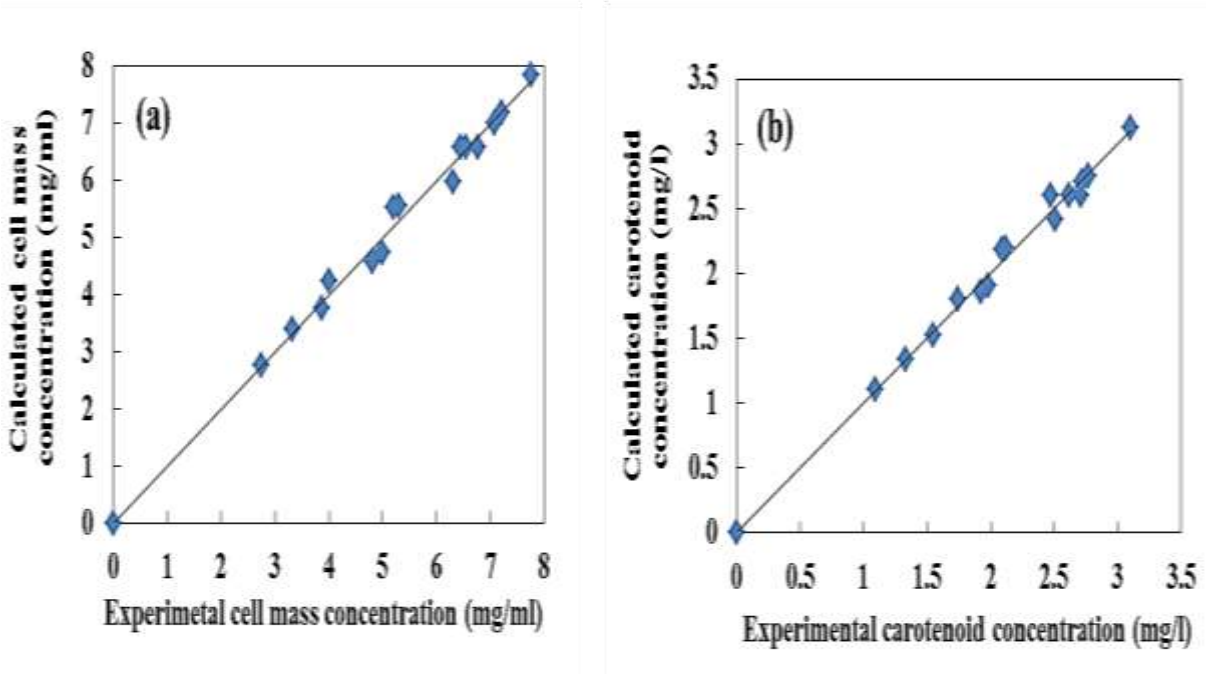


Fig. 4.4.3. (a) Cell mass concentration calculated from regression model equation versus the corresponding experimentally obtained values. (b) Carotenoid production calculated from the regression model equation versus the corresponding experimentally obtained values.

The obtained results are in good agreement with the predicted values of pigment and biomass production with ~ 4 % and ~ 3.6 % deviations. Therefore the results are in agreement with the effectiveness of the response surface approach described here. Furthermore, the obtained pigment yield was found to be in comparison with the earlier reports which employed sugar cane molasses [6] and mug bean wastes [13]. In addition to the higher yield, the economic aspects (simple methodology, cheap substrate) suggested that the pigment production can be scaled up to the industrial level without any special conditions. Till date, most of the pigment production was associated with the special conditions like acid hydrolysis of their substrate which may hinder the scale up to industrial level.

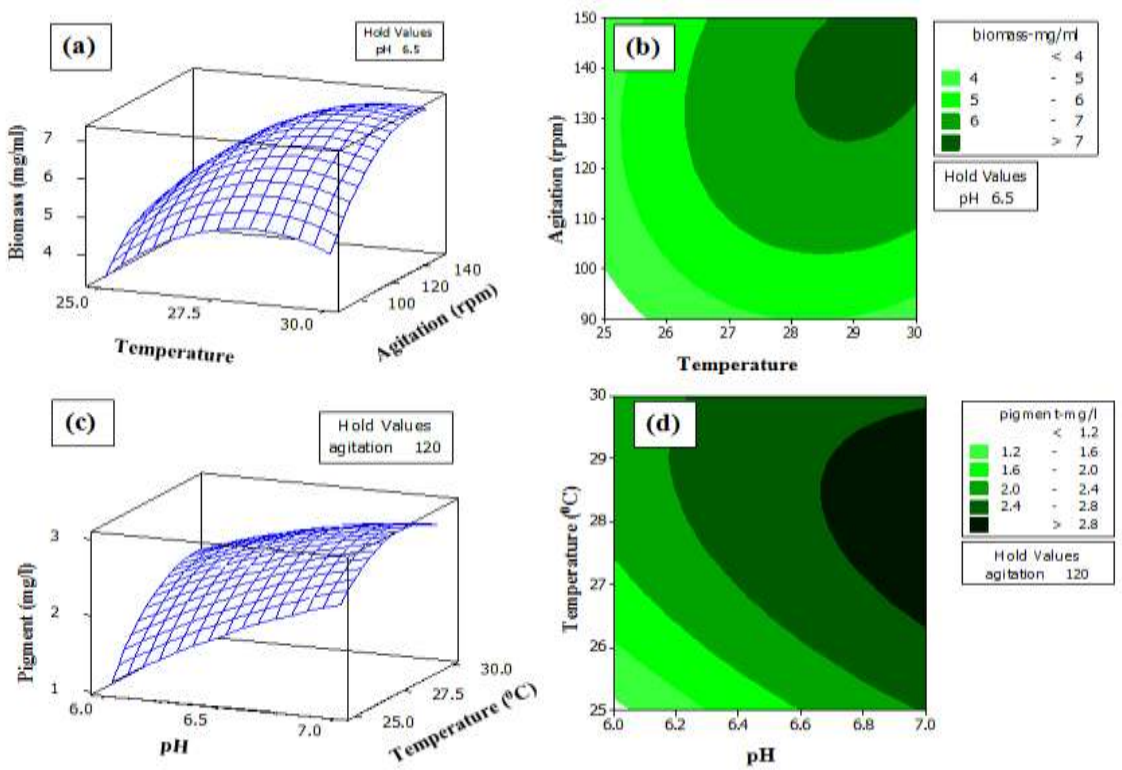


Fig. 4.4.4. Response surface and contour plots obtained from Equation (2) and (3) showing the effect of the temperature, agitation and their mutual interaction on biomass concentration (a), (b); while it is effect of pH and temperature and their mutual interaction on caroteniod pigment concentration (c), (d). The displayed units of all the graphs are in natural units.

4.4.3.4 DPPH radical scavenging activity

DPPH is a stable free radical that displays maximum absorbance at 517 nm. When DPPH radicals come across a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [22]. The decrease in absorbance is taken as a measure for radical-scavenging action.

After 40 min of incubation, free radical scavenging property with different doses (0 to 100 µg/mL) of *R. rubra* craotenoid was compared with ascorbic acid as a control (Fig. 4.4.5). A ~70 % scavenging activity of DPPH was achieved by ~100 µg/mL ascorbic acid, while it is only 60 % with the produced *R. rubra* craotenoid. Thus the carotenoid obtained was not a dominant radical scavenger with ~6 % lesser ability than the commercial ascorbic acid.

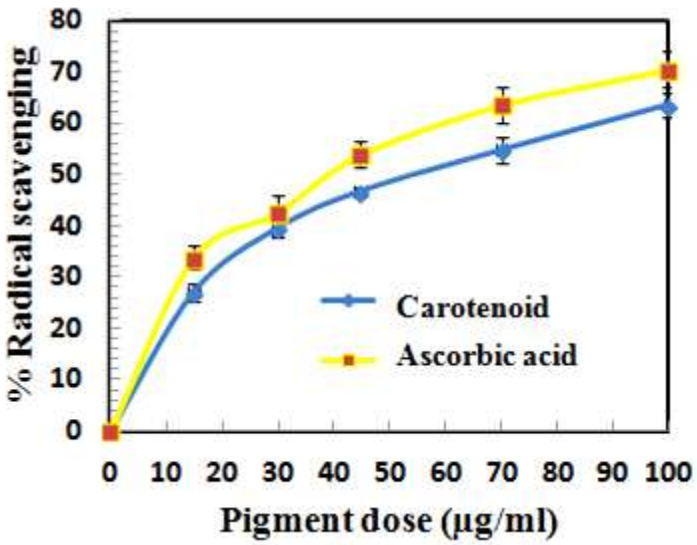


Fig. 4.4.5. Free radical scavenging property of the carotenoid by *R. rubra*. Dose dependent scavenging activity of the pigment compared with ascorbic acid (control).

4.4.4 Conclusions

In summary, the results obtained from the *R. rubra* in this study will be useful for efficient carotenoids production by utilizing the FWE as a sole substrate. The study also screened out key vital parameter like pH and optimized other imperative parameters such as temperature and agitation along with the pH using a response surface methodology. The simultaneous optimization of parameters yielded maximum carotenoid production conditions in an

economical way, for its large scale production using a cheaper substrate (FWE). Our results demonstrated that FWE could be profitably used as a suitable substrate without any additional growth supplement for noteworthy carotenoid production. The response optimization of parameters pH (7), temperature (28.2 ^{0}C) and agitation (150 rpm) by Box–Behnken design resulted in 51.2 % more enhancement of the mean carotenoid production. The produced carotenoid pigment is having potential antioxidant applications in food industries. This can be valued for industrial scale utilization of FWE to generate high value carotenoids using *R. rubra* and opens up scope for exploring other high value microbial pigments.

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4.5 Astaxanthin by *Xanthophyllomyces dendrorhous* Using Fruit Waste Extract as Sole Source of Energy: Optimization of Culture Conditions by Taguchi method for Improved Pigment Production

4.5.1 Introduction

Carotenoid class astaxanthin belongs to the group of lipophilic tetraterpenes, which are combination of eight isoprene units (C_5) that has important application in the neutraceutical, cosmetics, food and feed industries due to its high antioxidant activity [1-3]. Astaxanthin synthesized chemically contains only 8% astaxanthin and costs \$25,000-30,000 Kg^{-1}[4]. Synthetic astaxanthin is the major source of carotenoid currently being used in fish feeds [5]. However, food and feed additives from biotechnological processes are preferred by consumers to those produced by chemical technologies [6]. Among the microorganisms *Brevibacterium* [7], *Mycobacterium lacticola* [8], *Agrobacterium auratium* [9], *Haematococcus pluvalis* [10] and *X. dendrorhous* [4, 11] can be used for commercial

production of astaxanthin as 80-90% of the total carotenoids produced by these yeasts accounts for astaxanthin.

Among all red pigmented heterobasidiomycetous yeast, *X. dendrrorhous* has the potential to produce carotenoids on cheaper substrates like different agro-industrial raw materials with high yields [12]. This yeast strain has ability for the assimilation and metabolizing mono, di and polysaccharides, organic acid and alcohols. Moreover till date research is focused on this versatile strain on various cheaper substrates for significant astaxanthin production. Aiming towards cost cutting strategies, substrates such as waste streams like those from sugar manufacturing processes or the corn wet milling industry, white grape juice, enzymatic eucalyptus wood hydrolyzates [13], hemicellulosic hydrolyzates of eucalyptus globulus [14], yucca medium (based on date juice) [15], peat hydrolysate [16], corn steep liquor [17] etc. were successfully employed for the production of significant carotenoid class astaxanthin.

Very limited research has been carried out so far on fruit wastes and their extracts as sole source of energy for astaxanthin production using *X. dendrrorhous*. India contributes to 10 % of the world's fruit production [18]. Over last few years research focus is towards reprocessing and reuse of various fruit wastes for the conversion of nutritive and valuable products [19-20]. Previous studies on fruit wastes signify that it is a rich source of carbohydrates and other minor nutrients to support the microbial growth [19-20]. The surplus fruit wastes those are frequently disposed in the environment after exhaustive extraction of their juice may be possibly used as low cost substrates for production of microbial bioactive compounds like pigments. The availability of readymade simple sugars along with macro and micro nutrients make fruit waste as a suitable compound for the production of numerous

pigments by a variety of microorganisms. The above reported studies used special conditions and/or enriched the substrate media for high yield pigment production. In contrast, eying on cheaper, economical and quick synthesis process devoid of providing special conditions, we explored the capacity of readily prepared fruit waste extract (FWE) as sole substrate for astaxanthin production by acquired *X. dendrrorhous*. Thus this paper outlined the optimum production conditions for carotenoid pigment by *X. dendrrorhous* on FWE using Taguchi method.

4.5.2 Materials and methods

4.5.2.1 Chemicals and microorganism

Acetone, ascorbic acid, methanol, NaCl, HCL and diethyl ether were procured from Merck, India. The strain *Xanthophyllomyces dendrorhous* (7536) was obtained from MTCC Chandigarh. The yeast was maintained on Yeast extract/ malt extract (YM) media agar plates at 4 ^{0}C and sub cultured monthly in YM media broth. YM media and agar were purchased from HiMedia chemicals, India.

4.5.2.2 Substrate preparation

Fruit waste is obtained from local juice shop from local market, Rourkela, Odisha, India, which includes pineapple waste, orange waste and minor portion of pomegranate waste. Fruit waste extract (FWE) media is prepared from mixed waste by boiling and extracting the soluble sugars for 30 min using distilled water.

4.5.2.3 Culture conditions

The inoculum was prepared in YM broth at 22 ^{0}C for 48h. The yeast cells were harvested by centrifugation and washed twice with distilled water. 1 mL of microbial solution (1 % w/v)

was inoculated in a 250 ml Erlenmeyer flask containing 50 ml FWE medium. The inoculated cultures were kept at 22 ^{0}C in incubator shaker at a shaking speed of 150 rpm for pigment production. Yeast extract/malt extract (YM) medium is used as the reference medium for comparison with FWE medium. Each experiment was performed thrice to minimize the error.

4.5.2.4 Pigment production and extraction/ Sugar determination

Yeast cells obtained after centrifugation was washed twice with distilled water. Pigmented cells were treated with methanol: acetone (1:1) solvents and centrifuged. This process was repeated until colorless biomass was obtained. The extracted pigment was subjected to phase separation using equal volumes of petroleum ether and 10 % NaCl. The carotenoid was collected from the diethyl ether phase and analyzed at 474 nm by UV-visible spectrophotometer using an extension coefficient of $A_{1\ cm}^{1\ \%}$ = 2,100 [21]. Obtained carotenoids were further purified using thin layer chromatography using stationary phase - TLC silica gel 60 F_{254} and hexane: methanol (7:3) as stationary phase and solvent phase respectively. The purified pigment was further analyzed in FTIR to determine the functional groups.

The 3,5- dinitrosalicyclic acid (DNS) method of miller [22] was used to determine the reducing sugars in FWE medium. About 1ml sample was centrifuged at 3,500 X g for 5 min, and 1 mL DNS reagent was added to the supernatant and boiled for 5 min. The sample was placed in ice –bath for rapid cooling. Distilled water was added to the samples and vortexed for 5 min. The optical density was calculated at 575 nm. In addition, CHNS analysis was performed before and after the pigment production

4.5.2.5 Taguchi design and statistical analysis

Simple Taguchi orthogonal array design was selected where there are three factors and three levels. From the reported studies, pH (4-6), temperature (15-25 ^{0}C) and agitation (100-300

rpm) were found to be significant in obtaining higher amounts of astaxanthin production by *X. dendrorhous* [23-25]. In this study Taguchi method was used for optimization of pigment production conditions. Optimization of stated factors in three different levels is illustrated in Table 4.5.1. To perform the Taguchi method, 9 different experiments using L9 orthogonal array was run as shown in Table 4.5.2. Based on the primary results a verification test was employed to check the optimum condition. An analysis of variance (ANOVA) for the obtained results was examined. Design of experiments, ANOVA and optimization of process were accomplished using MINITAB- 14 software.

4.5.2.6 Determination of free radical scavenging activity

The effect of astaxanthin from *X. dendrorhous* on DPPH free radical was studied as per the method of Gramza-Michałowska and Stachowiak[26] with some modifications. A 2 mg/ml pigment suspension was prepared in varying volumes of ethanol (10 to 60 µl). To the suspension 1 ml DPPH solution containing 0.1 mM DPPH and 99.5 % ethanol (99.5 %) was added. The mixture was thoroughly mixed and allowed to stand for 40 min at room temperature in the dark. Ascorbic acid is used as positive control and the pigment solution without DPPH as negative control. The radical scavenging activity was measured as a decrease in the absorbance of DPPH at 517 nm wavelength and was calculated using the following formula:

$$\%\ \text{Scavenging activity} = \frac{(\text{A}c - \text{A}s)}{(\text{A}c)} \times 100$$

Where A_c, A_s are absorbance's from control and sample.

4.5.2.7 Analytical methods

UV-visible spectra and radical scavenging activity were measured using an UV-visible-NIR spectrophotometer (Shimadzu, UV-3600). Morphological characteristics of the used microorganism was studied by using scanning electron microscopy (SEM, JEOL JSM 6480 LV), active functional groups of the produced pigment was identified through Fourier infrared spectroscopy (FTIR, Bruker, USA) equipped with a horizontal attenuated total reflectance (ATR) device with zinc selenide (ZnSe) crystal.

Table 4.5.1. Factors and their levels studied by Taguchi method.

Factor	*Level 1*	*Level 2*	*Level 3*
pH	4	5	6
Temperature (^{o}C)	15	20	25
Agitation (rpm)	100	200	300

Table 4.5.2. Levels of three different factors applied in each of nine trials, with observed results.

pH	Temperature(^{O}C)	Agitation (rpm)	Astaxanthin (mg/g)	Biomass (g/L)
4	15	100	0.402	0.3216
4	20	200	0.556	0.548
4	25	300	0.525	0.62
5	15	200	1.24	1.133
5	20	300	1.252	1.34
5	25	100	0.91	0.728
6	15	300	0.978	1.28
6	20	100	0.88	0.704
6	25	200	1.211	1.103

4.5.3 Results and discussion

Among various yeasts, *X. dendrorhous* has been exploited as major source of astaxanthin with potential commercial applications in the fields of cosmetics, pharmaceuticals and agriculture [5, 7]. However, astaxanthin production from cheaper substrates has limited reports. So optimization of its process parameters for astaxanthin production has been extensively studied to emphasize its production in commercial scale. Substrate utilization/growth and pigment production by yeast was significantly influenced by key factors like pH, temperature and agitation; hence these factors play a vital role in the cost effectiveness of pigment production. The pigmentation of the FWE and the SEM image of the studied yeast were given in Fig. 4.5.1.

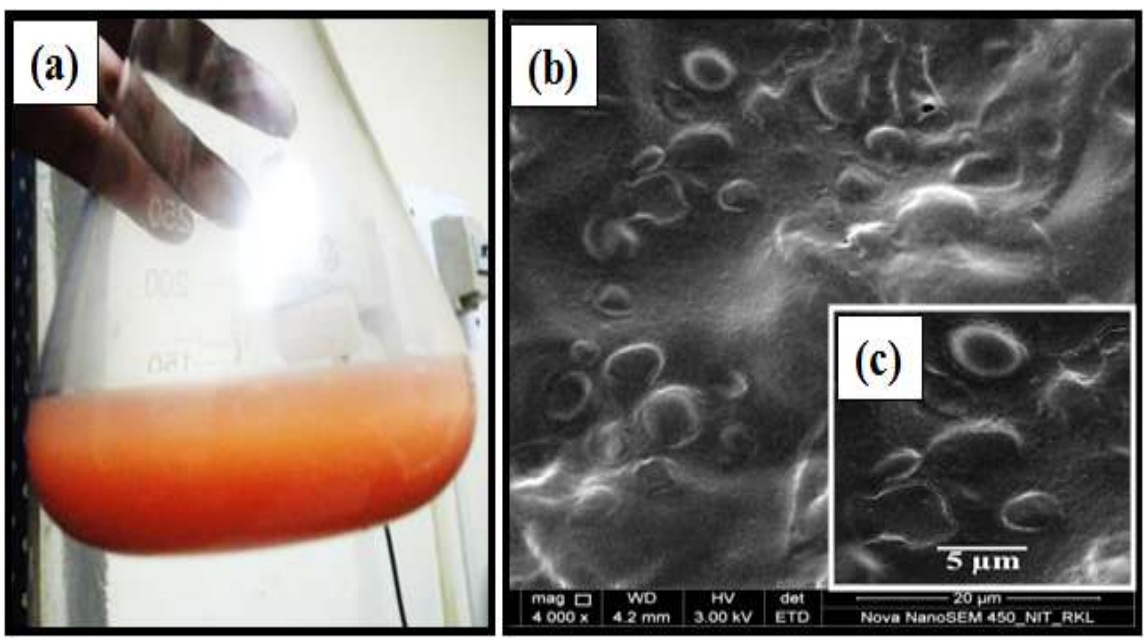

Fig. 4.5.1. Pigmentation of FWE (a) by before *X. dendrorhous* yeast with lower (b) and higher (c) magnification SEM images.

4.5.3.1 Experimental design by Taguchi method

Effects of pH, temperature and agitation were studied using Taguchi method, which is a fractional factorial experimental design. The results of experiments performed in this section (Table 4.5.2) showed that the maximum average yield of pigment 1.252 mg/g, at experimental conditions pH 5, temperature 20 ^{o}C and agitation 300 rpm. Fig. 4.5.2 depicts the main effect of each of these factors that implies the average results obtained for each factor in order to screen out significant factors the statistical technique of analysis of variance (ANOVA) with F test was employed to the simulation reflecting the data shown in Table 4.5.3. The ANOVA results implicates from F value of 23.71 that pH has significant effect on pigment production. Similarly temperature showed negligible effect. Thus the optimum experimental condition was selected from the obtained result as pH 5, temp 20 ^{o}C and agitation 300 rpm. When the results were analyzed, an optimum condition was proposed by calculations.

4.5.3.2 Validation of the model

Table 4.5.4 shows the suggested conditions for maximum pigment production. Statistical calculations predicted that if the conditions were chosen as shown in Table 4.5.4, the pigment production should reach 1.264 mg/g biomass. However after performing the experiment at said condition, the produced astaxanthin was 1.40 ± 0.14 mg/g. The difference between predicted and actual result was about 10 %, and is regarded as acceptable and promising.

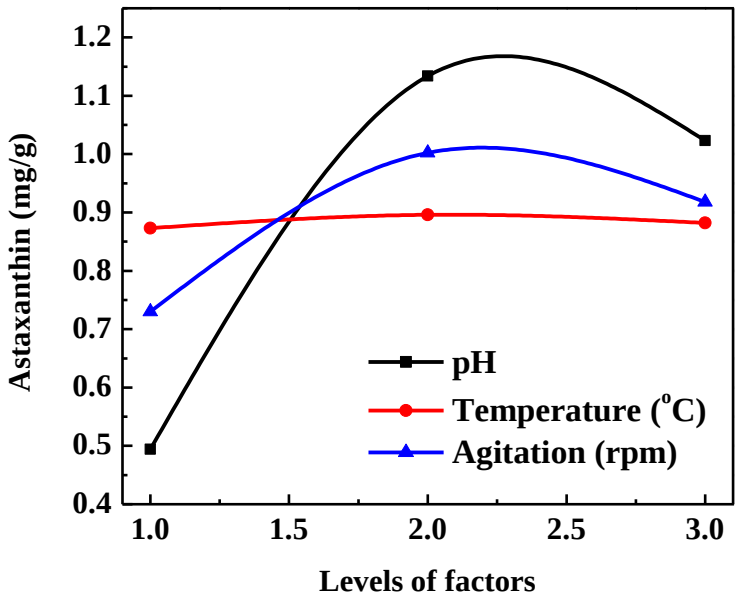

Fig. 4.5.2. Main effects of factors or average of obtained results as mg/g biomass in which each factor is at a given level. For description of 'levels' refer to Table 4.5.1.

Table 4.5.3. Analysis of variance of main effects of factors

Analysis of Variance for Means

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	2	0.7009	0.7009	0.3504	23.71	0.04
Temperature	2	0.0007	0.0007	0.0003	0.03	0.974
Rpm	2	0.1160	0.1160	0.0580	3.93	0.203
Residual Error	2	0.0295	0.0295	0.0147		
Total	8	0.8474				

Table 4.5.4. Optimum conditions suggested by statistical calculations after performing the experiments.

Factor	Level description	Level	Contribution in 'mg/g'
pH	5	2	1.134–0.883=0.251
Temperature (^{o}C)	20	2	0.896 –0.883=0.013
Agitation (rpm)	200	2	1.00 –0.883=0.117

4.5.3.3 Pigment yield, biomass trend and glucose utilization

Table 4.5.4, illustrates the optimum condition for astaxanthin pigment production. Growth kinetics of the pigment production, cell mass and substrate utilization has been studies at optimum experimental condition as represented in Fig. 4.5.3a. The figure shows that both biomass and astaxanthin production increased over time as total soluble sugars content decreased. The maximum levels of biomass (~22.4 g/L) and astaxanthin (~31.5 mg/L) were reached at 84 and 96 h, while the carbon source (soluble sugars) had been depleted and reaching a constant level from 96 to 144 h (Fig. 4.5.3a).

From the figure, the time course of the biomass growth showed the characteristic exponential and stationary phases and reached a maximum level after about 84 h. Moreover it was also noted that the pigment production was not constant at stationary phase of biomass growth and went on increased up to 108 h. The observed behavior infers the ability of the used strain to give significant amounts of astaxanthin even in stationary phase, which is beneficial effect of the used substrate (under the used conditions).

From this result it is clear that the astaxanthin production was partially dependent on biomass growth and still occurred even after growth and sugars depletion. Such behavior is

certainly related to the fact that *X. dendrorhous* excretes and stocks some extracellular carbon intermediates, which stimulate late carotenogenesis [7].

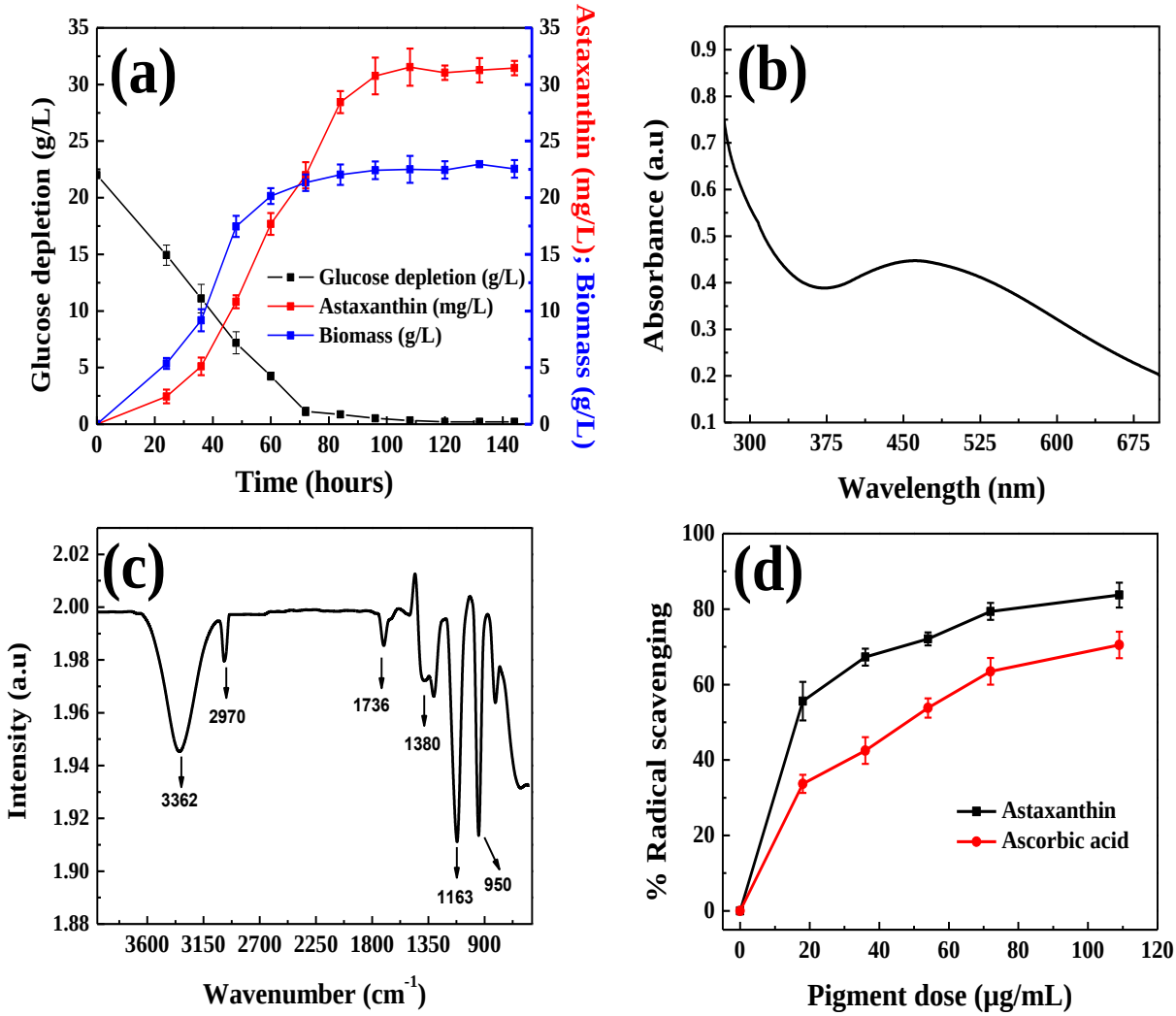

Fig. 4.5.3. (a) Time course of the growth and production of astaxanathin by *X. dendrorhous* in FWE. Experiment was carried out at optimum conditions i.e. pH (5), temperature (20 ^{o}C) and agitation (300 rpm). (b) UV-Visible and FTIR spectrum (c) of the produced pigment in methanol. (d) Antioxidant activity (DPPH radical scavenging) of astaxanthin and ascorbic acid.

4.5.3.4 UV analysis

The spectral property of the produced astaxanthin in FWE was shown in Fig. 4.5.3b. The absorption maximum was identified to be at 474 nm in methanol and was in good agreement with the reported value [27].

4.5.3.5 FTIR analysis

The purified pigment from the TLC plate was dissolved in ethanol and analyzed for FTIR spectral analysis and the results are shown in (Fig. 4.5.3c). According to Coates et al. [28] the peak descriptions of the FTIR spectra can be described as follows: The peak at 3362 shows the presence of O-H hydrogen bond. The peak at 2970 is due to methyne C-H stretch; a peak at 1736 shows the presence of C=O bond in the molecule. The peak at 1380 is due to methyl C-H asymmetric band. The peak at 1163 is for C-O stretch and the peak at 950 represents the skeletal vibrations. The spectral behavior of the pigment closely resembles the structure of astaxanthin which is predominantly obtained by *X. dendrorhous* [29].

4.5.3.6 DPPH radical scavenging activity

DPPH is a stable free radical that displays maximum absorbance at 517 nm. When DPPH radicals come across a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [30]. The decrease in absorbance is taken as a measure for radical-scavenging action. The DPPH radical-scavenging activity was investigated at different concentrations (0 to 109 µg/mL) of the produced astaxanthin. The results are shown in Fig. 4.5.3d.

After 40 min of incubation, it could be observed that the radical scavenging activity increased promptly with an increase in astaxanthin dose when compared to ascorbic acid (control) and reached up to ~85 % at the dose of 109 µg/ml. However, it is also noted that ascorbic acid helped in achieving only ~70 % under the same conditions. Although astaxanhin and ascorbic acid both showing an increase in % radical scavenging, it is noteworthy that the former was higher than the latter at any moment within the studied pigment dose (Fig. 4.5.3d).

In summary, the key factors that independently influence biomass growth and pigment production were identified by Taguchi method. At identified key parameters, FWE appears to be an excellent substrate since a remarkable astaxanthin production (1.4 ± 0.14 mg/g biomass) was obtained when compared to YM medium i.e. 1.66 ± 0.21 mg/g biomass at same culture conditions. YM is an expensive source for astaxanthin production with C to N proportion of 1.0 and 0.3 %, while FWE is an inexpensive alternative with C to N proportion found to be 1.0 and 0.066 % (observed by CHNS analysis). This work shows that FWE medium plays a key role in astaxanthin production by *X. dendrorhous* and it is worth noting that, in our case we achieved enhanced astaxanthin production with free radical scavenging activity without any substrate supplements and special conditions.

4.5.4 Conclusions

This study shows the suitability of the FWE medium for the production of astaxanthin by *X. dendrorhous* yeast strain. Critical parameters screened by simple Taguchi approach and optimum conditions for the enhanced production of pigment were established. Increase yield was observed at pH - 5, Temp - 20 °C and Agitation-200 rpm with optimum production of

1.4 ± 0.14 mg/g biomass. UV-visible and IR analysis of the purified pigment illustrated the structural confirmations of the pigment. In addition, free radical scavenging activity of the obtained pigment gave 15 % better activity than ascorbic acid and encourages its applicability as a constitutional ingredient in cosmetics.

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Chapter 5 Conclusions and future perspectives

Conclusions

The main outcome of this work is that we have screened some novel potent pigment producing microorganisms, bacteria in particular, which can produce melanins and carotenoids on cheaper and renewable substrates in high amounts. This work also explores the pigment producing ability of the obtained microbial strains (yeasts) on various cheaper substrates and accomplished noticeable high yields on a fruit waste extract medium. The beauty of the work lies in the identification of the key parameters of pigment production (for isolated as well as obtained strains) and optimization of the influential parameters for improved pigments production.

- The present exertion starts with the production of melanin by a marine isolate in part I-4.1 of Chapter 4. Here marine bacterium capable of melanin production on marine broth/agar was isolated and identified as *Pseudomonas* sp. (closely related to *guinea*) on phenotypic characterization. Melanin production activity of the isolate was studied in liquid media such as pure marine broth and vegetable cabbage waste. In pure marine

broth, melanin yield was ~5.35 mg/mL and pigment production was negligible in pure vegetable waste medium. However in the presence of marine broth (as starter culture) melanin yield increased to ~2.79 mg/mL. This indicates melanin production may be initiated austerely by marine broth. The characterized melanin was very near to synthetic dihydroxyphenylalanine (DOPA)-melanin in all aspects. Furthermore, the purified melanin upon analyzing for sun protection activity showed promising result. SPF value of the melanin here was found to be 53.74 where, it was 59.34 for synthetic melanin. Additionally radical scavenging activity of the obtained melanin was higher than the ascorbic acid (control) at all doses which showed 40% of enhanced reductive capability. ~55% ferrous ion chelation activity was noticed for the dose of 0.146 mg/mL which was greater than synthetic melanin.

- The pigment outcome from a soil microbial isolate addressed in part I–4.2 of Chapter 4 was a melanin and abundantly produced on a fruit waste extract medium. The garden soil isolate here was identified to be *Bacillus safensis*. The optimization of process parameters influencing melanin production was attempted using a simple two step methodology. Taguchi approach was adopted for screening of critical parameters for pigment production, and optimization study was performed using the response surface methodology (RSM) with a central composite design. At optimum conditions of pH - 6.84 and Temp - 30.7 ºC, a significant yield of 6.96 ± 0.6 mg/mL was observed. Statistical analysis revealed that the experimental results fitted well to this statistical model with model R^2 value 0.982. The optimization of process parameters using RSM reported a 15 % increase in the yield of melanin than average yield by the trails of the studied model. The melanin pigment obtained here too exhibited significant

photoprotective capability with sunscreen protection factor 53.36, while it was 59.34 for synthetic melanin. It also showed ~ 30% greater radical scavenging activity than ascorbic acid for a constant melanin dose of 100 µg/mL. and metal chelating activity studies showed that obtained melanin has maximum chelation ~ 64 % for a dose of 0.2 mg/mL.

- Microbial method of carotenoid pigment (β-carotene type) production was attained by *Bacillus clausii* (a garden soil isolate) using rice powder as the sole substrate in part I-4.3 of Chapter 4. Influence of substrate (rice powder) on β-carotenoid production has been extensively studied at optimum process conditions such as pH, temperature etc. The maximum yield of β-carotenoid 48.9 % using *B.clausii* was achieved at pH 7 and 35 ^{o}C utilizing rice powder as a sole substrate. A statistical design technique, Taguchi method was applied to evaluate the optimal process conditions such as pH, temperature etc. for maximum pigment production.
- The investigations of part II-4.4 and 4.5 of Chapter 4 shows the effectiveness of abundantly available cheaper fruit waste extract as a sole substrate in obtaining carotenoids in significant amounts by *R. rubra* and *X. dendrorhous* yeast strains. In 4.4, a two-step simple sequential strategy was employed for the optimization of carotenoid production by *R. rubra*. In the first step, one factor at a time was employed to evaluate the impact of pH on carotenoid production. The outcome revealed that pH has a noteworthy influence on pigment production at ambient conditions with constant temperature and agitation. A Box–Behnken design was then applied in the second step to optimize the pH, temperature and agitation to obtain high pigment yield. The statistical experimental design predicted the high yield conditions of different responses. The

interaction between pH and temperature stood vital for improved carotenoid production (2.98 ± 0.28 mg/l) with biomass yield of 7.83 mg/ml by the optimization of significant parameters. The optimum conditions followed for high yield carotenoids are pH (7.0), temperature (28.2 ^{0}C), and agitation (150 rpm). The purified pigment upon testing for free radical scavenging activity displayed moderately comparable and ~6 % lesser ability than the tested ascorbic acid as a control.

- While addressing the later study in 4.5, Taguchi method was employed to find the optimum pigment production conditions of the yeast in FWE medium. Effect of key parameters (pH, temperature and agitation) was investigated using L9 orthogonal array by three factor-three level approach and significant parameters influencing pigment yield was checked by analysis of variance. Key parameter's contribution was suggested by statistical calculations and assessed by a validation test. Conducting the experiments at optimum conditions (pH-5, Temp-20 ^{0}C and Agitation-200 rpm), an increase in 10 % astaxanthin was achieved. This study shows a simple and cost effective synthesis of astaxanthin by *X. dendrorhous* using FWE. Additionally free radical scavenging activity of the purified pigment gave 15 % better activity than the ascorbic acid with a constant dose of 100 µg/mL.

The above evidences suggest that, FWE has huge potential to be an economical substrate for various microorganisms for novel pigments production with no medium enrichments and special conditions. The established optimized conditions in FWE have huge scope to be scaled-up for the large scale production of melanins and carotenoids by selective microorganisms which are having significant applications in cosmetics, agriculture and medicine sectors.

Future Perspectives

- A systematic screening of other microbial habitants for pigment producing microbes and their exploitation of a wide range of different cheaper substrates is highly recommended.
- Evaluation of potent pigment producing organisms like fungi and bacteria has to be exploited on different inexpensive materials as a sole source of substrates will warrant pigments production economical.
- Robust Taguchi and response surface approaches should be implemented during the cultivation procedures to identify the key process parameters and to develop a model based production strategy. Statistical methods not only save time, but also eliminate the wastage of media and materials.
- Toxicity issues to be thoroughly studied for the novel identified pigments by developmental stage for marketability and consumer acceptance.

Publications from the work

- Tarangini Korumilli and Susmita Mishra, Production of Melanin by Soil Microbial Isolate on Fruit Waste Extract: Two Step Optimization of Key Parameters. *Biotechnology Reports*, 4, 39-146 (2014).
- Tarangini Korumilli and Susmita Mishra, Carotenoid Production by *Bacillus clausii* Using Rice Powder as the Sole Substrate: Pigment Analyses and Optimization of Key Production Parameters, *Journal of Biochemical Technology*, 5, 788-794 (2014).
- Tarangini Korumilli and Susmita Mishra, Carotenoid production by *Rhodotorula* sp. on fruit waste extract as a sole source and optimization of key parameters. *Iranian journal of Chemistry and Chemical Engineering*, 33, 97-106 (2014).
- Tarangini Korumilli and Susmita Mishra, Production, Characterization and Analysis of Melanin from Isolated Marine *Pseudomonas sp*. using Vegetable waste, *Research Journal of Engineering Science*, 2, 40-46 (2013).
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CURRICULUM VITAE

TARANGINI KORUMILLI
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Career Goal

To work in a challenging academic position that gives me an opportunity to prove myself and to be an outstanding asset to the organization in which I work.

Current status

Doctorial Research scholar engaged in ***"Microbial pigments"*** research, especially on ***"Exploring simple and economical methods of production of microbial pigments on cheaper substrates and investigating their biological applications".***

Academics

Course	College/University	Year of Passing	Class / % of Marks
M.Tech (Res) (Chemical Engg)	National Institute of Technology, Rourkela, Orissa, India.	2009	**First Class**/ (86.80 %)
B.Tech. (Bio-Technology)	Godavari Institute of Eng & Technology, JNTU, (Rajahmundry, A.P, India)	2006	**First Class**/ (76.40 %)
Intermediate (Bi.P.C)	Aditya Junior College, (Board Of Intermediate Education, A.P.)	2001	**First Class**/ (80.50 %)

Areas of Interest

Biochemical engineering, Food science and technology, Biosorption, Molecular Biology, Genetics, Bioprocess Engineering, Immunology, and associated areas of Biotechnology.

Skill Profile

- Expertise with instruments like TGA, AAS, UV-visible spectroscopy, Sonicator, Spray drier, HPLC etc. with all basic analytical techniques.

Other skills: System languages C; Operating System-Windows XP, Windows 7

Assets

- Good communication, practical & positive thinking, interpersonal and leadership skills. Thrive in both independent and collaborative work environments.
- Quick learner with an ability to rapidly achieve organizational integration, assimilate job requirements and employ new ideas, concepts, methods and technologies
- Efficient in handling projects and a team.

Major Achievements

International Publications

- **Tarangini Korumilli** and Susmita Mishra, Production of Melanin by Soil Microbial Isolate on Fruit Waste Extract: Two Step Optimization of Key Parameters. *Biotechnology Reports*, 4, 39-146 (2014).
- **Tarangini Korumilli** and Susmita Mishra, Carotenoid Production by *Bacillus clausii* Using Rice Powder as the Sole Substrate: Pigment Analyses and Optimization of Key Production Parameters, *Journal of Biochemical Technology*, 5, 788-794 (2014).
- **Tarangini Korumilli** and Susmita Mishra, Carotenoid production by *Rhodotorula* sp. on fruit waste extract as a sole source and optimization of key parameters. *Iranian journal of Chemistry and Chemical Engineering*, 33, 97-106 (2014).
- **Tarangini Korumilli** and Susmita Mishra, Production, Characterization and Analysis of Melanin from Isolated Marine *Pseudomonas sp*. using Vegetable waste, *Research Journal of Engineering Science*, 2, 40-46 (2013).
- **Tarangini Korumillii**, Arvind Kumar, G. R. Satpathy, Vikas Kumar, SangalStatistical optimization of process parameters for Cr (VI) biosorption onto mixed cultures of **Pseudomonas aeruginosa** and **Bacillus subtilis, *CLEAN - Soil, Air, Water*, Wiley Inter Science. 37, 319-327 (2009).**
- **Tarangini Korumilli** and G. R. Satpathy, Optimization of heavy metal biosorption using attenuated cells of *Bacillus subtilis* and *Pseudomonas aeruginosa*. ***Journal of Environmental Research and Development*, 3, 677-684 (2009).**
- **Tarangini Korumilli** and Susmita Mishra, Astaxanthin by *Xanthophyllomyces dendrorhous* Using Fruit Waste Extract as Sole Source of Energy: Optimization of Culture Conditions by Taguchi method for Improved Pigment Production. ***Biotechnology Reports - Submitted (In review) 2014.***

National and International Conferences

- **Presented a paper in** International Congress on Environmental Research and Development (ICER-08) **on** Optimization of heavy metal biosorption using attenuated cells of *Bacillus subtilis* and *Pseudomonas aeruginosa*. 2008.
- Secured **second place** in **National Level Paper Presentation** on ***Solar Photo catalyzed Oxidation Reactions using TiO_2 for killing bacteria*** at S.N.E.T – 2003.
- Secured **second place** in **State Level Paper Presentation on** ***Bio Hydrogen Production from Rice Bra Oil Mill waste*** at Srujana – 2005.
- Participated in **National Level Symposium**, held in **Vellore Institute of Technology on** ***Bio Hydrogen Production from Paper Mill waste*** – 2005.
- Participated in **National Level Symposium**, held in Ananthapur J.N.T.U on **A *Non – Conventional approach for Bio Hydrogen Production* -**2005.
- Presented a Paper at **National Seminar on** ***Advances in the frontiers of environment research at Andhra University*** – Nov 2005.
- Working Model on ***Microbial analysis of soil in and around Rajahmundry*** at Srujana – 2004.
- Working Model on ***Mushroom cultivation*** at Srujana – 2005.

Academic Projects

Title	**"Optimization of Heavy metal Biosorption using attenuated cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*."**
Project theme	Eco-friendly and cost effective removal of heavy metals from the aqueous solutions using Biosorption technique
Application of work	Today's world is facing serious problem of environmental pollution where water pollution occupies a major place. So, to reduce this water pollution we are removing dissolved hazardous and carcinogenic metals by Biosorption technique by using individual and mixed cultures of *Bacillus* and *Pseudomonas species*.

Personnel Details

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East Godavari District, Andhra Pradesh, Phone: 08852 – 244355.

References **:** Available on request